DNA Packaging and Host Cell Lysis: Late Events in Bacteriophage PRD1 Infection

Gabija Žiedaitė

Institute of Biotechnology and Department of Biological and Environmental Sciences
Division of General Microbiology
Faculty of Biosciences
and
Viikki Graduate School in Molecular Biosciences
University of Helsinki

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biosciences, University of Helsinki, in auditorium 3 of Info center Korona, Viikinkaari 11, Helsinki, on 7th of November 2008, at noon.

HELSINKI 2008

Supervisors

Professor Dennis H. Bamford
Institute of Biotechnology and
Department of Biological and Environmental Sciences
University of Helsinki
Finland

Professor Jaana K. H. Bamford
Department of Biological and Environmental Sciences
University of Jyväskylä
Finland

Reviewers

Docent Mikko Frilander
Institute of Biotechnology
University of Helsinki
Finland

Docent Roman Tuma
Astbury Centre for Structural Molecular Biology
Institute of Molecular and Cellular Biology,
Faculty of Biological Sciences,
University of Leeds
UK

Opponent

Doctor Paulo Tavares
Unité de Virologie Moléculaire et Structurale,
Centre National De La Recherche Scientifique (CNRS),
Paris
France

Cover picture: courtesy of Dr J. Cockburn

ISBN 978-952-10-5019-0 (paperback)
ISBN 978-952-10-5022-0 (PDF, http://ethesis.helsinki.fi)
ISSN 1795-7079

Yliopistopaino, Helsinki University Printing House
Helsinki 2008

To those who believed

Original publications

This thesis is based on the following articles that are referred to in the text by their respective Roman numeral.

I Karhu, N. J., Žiedaitė, G., Bamford, D. H. & Bamford, J. K. H. (2007). Efficient DNA Packaging of Bacteriophage PRD1 Requires the Unique Vertex Protein P6. *J. Virol.* **81**:2970-2979.

II Žiedaitė, G., Kivelä, H. M., Bamford, J. K. H. & Bamford D. H. (2008). Purified membrane-containing procapsids of bacteriophage PRD1 package the viral genome. *Submitted.*

III Žiedaitė, G., Daugelavičius, R., Bamford, J. K. H. & Bamford, D. H. (2005). The Holin Protein of PRD1 Forms a Pore for Small-Molecule and Endolysin Translocation. *J.Bact.***187**:5397-5405.

Abbreviations

aa	amino acid
ATP	adenosine triphosphate
bp	base pair
C-	carboxyl (when in polypeptide)
CM	cytoplasmic membrane
DNA	deoxyribonucleic acid
ds	double stranded
EM	electron microscopy
HBV	Hepatitis B virus
HSV-1	Herpes simplex virus 1
IHF	*Escherichia coli* integration host factor
IPTG	isopropyl-β-D-thiogalactopyranoside
ITR	inverted terminal repeat
kbp	kilobase pairs
kDa	kilodalton
LIN	lysis inhibition
Mpf	Mating pair formation
N-	amino (when in polypeptide)
nt	nucleotide
NTP	nucleoside triphosphate
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
pfu	plaque-forming unit
p.i.	post infection
pN	pico Newtons
pRNA	*p*rohead RNA or *p*ackaging RNA
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
ss	single stranded
sus	suppressor sensitive
TMV	Tobacco mosaic virus
TPP^+	tetraphenylphosphonium
wt	wild-type
$\Delta\Psi$	membrane voltage

Summary

The object of this study is a tailless internal membrane-containing bacteriophage PRD1. It has a dsDNA genome with covalently bound terminal proteins required for replication. The uniqueness of the structure makes this phage a desirable object of research. PRD1 has been studied for some 30 years during which time a lot of information has accumulated on its structure and life-cycle. The two least characterised steps of the PRD1 life-cycle, the genome packaging and virus release are investigated here.

PRD1 shares the main principles of virion assembly (DNA packaging in particular) and host cell lysis with other dsDNA bacteriophages. However, this phage has some fascinating individual peculiarities, such as DNA packaging into a membrane vesicle inside the capsid, absence of apparent portal protein, holin inhibitor and procapsid expansion.

In the course of this study we have identified the components of the DNA packaging vertex of the capsid, and determined the function of protein P6 in packaging. We managed to purify the procapsids for an *in vitro* packaging system, optimise the reaction and significantly increase its efficiency. We developed a new method to determine DNA translocation and were able to quantify the efficiency and the rate of packaging. A model for PRD1 DNA packaging was also proposed.

Another part of this study covers the lysis of the host cell. As other dsDNA bacteriophages PRD1 has been proposed to utilise a two-component lysis system. The existence of this lysis system in PRD1 has been proven by experiments using recombinant proteins and the multi-step nature of the lysis process has been established.

Table of contents

A. INTRODUCTION

1. Virus structure

Viruses are noncellular biological entities that use host cells for replication and are characterised by an extracellular state, the virion. A virion is a large macromolecular complex of an RNA or DNA genome and a protective outer layer, the capsid. During the course of evolution capsid structure developed to fulfil two major tasks: protection of the viral genome from damaging environmental factors and efficient delivery of the genome into the target cell. Capsids also contain structures for performing many other functions, including: host cell recognition and binding, genome injection to host cell, host cell lysis, and modulation of the host immune response. Viral capsids come in a variety of shapes and sizes. There are several shapes of capsids clearly favoured by evolution: helical–rod/filamentous, icosahedral, and capsids with no clear symmetry (Fig. 1). Amongst bacteriophages a binary (combined) symmetry is prevalent, as about 96 % of phages have an icosahedral head and a filamentous tail attached to one of the vertices of icosahedron (Ackerman 2006). The tailed phages (*Caudovirales*) can be further divided according to the types of their tails; which can be long and contractile (*Myoviridae*), long and rigid (*Siphoviridae*) or short (*Podoviridae*).

Many viral capsids in addition to nucleic acids and proteins also have a lipid bilayer. Usually this bilayer coats the surface of the protein capsid and is acquired when the virion buds out of the cell or out of the cellular organelles into the cytoplasm (Navaratnarajah et al., 2008). Some viruses have a lipid bilayer inside the protein capsid, such are phages of the *Tectiviridae* family (Bamford, 2005, Bamford & Butcher, 2008).

Viral genomes are also variable. They can be DNA or RNA, in single- or double-stranded form, circular or linear, with or without terminal proteins (Ackerman, 2005, 2006).

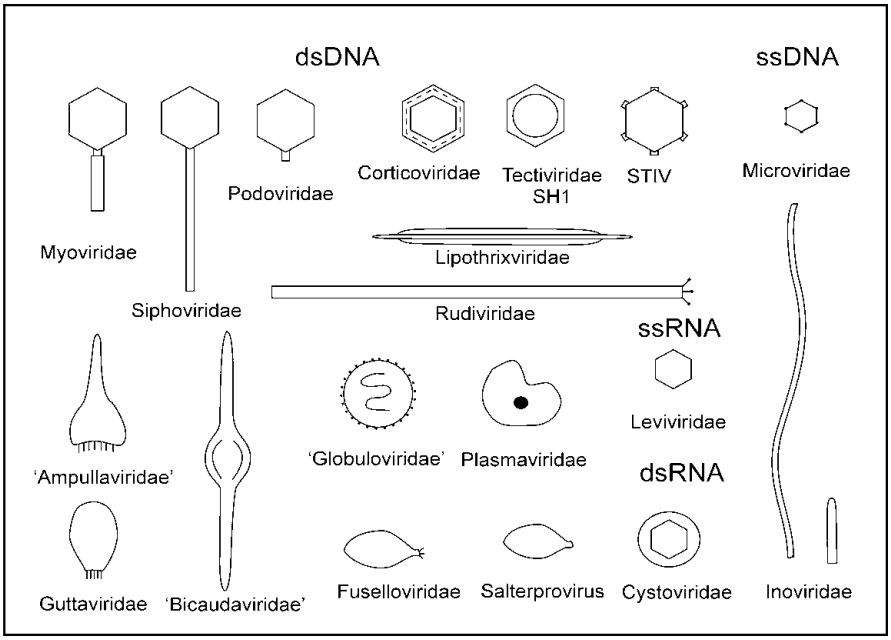

Figure 1. Morphotypes of bacteriophages and archeal viruses. Modified from Ackermann 2007.

2. Life cycle

Viruses have been described as intracellular parasites having an intracellular and extracellular state (Voyles 2002). The ‘living state’ is represented by the intracellular reproduction phase, when viruses use host cell resources and metabolism to produce progeny virions. The "nonliving state" of the virus is the virion on the prowl for a new host. The easiest way to study the whole cycle of interactions between the virus and the host cell is a one-step growth experiment (Adams 1959, Voyles 2002). This cycle can be divided into several different stages. The first stage is **attachment** or **adsorption** to the host cell. This step involves specific recognition events at the cell surface and requires specialized virus and cell structures. The next step is virus **penetration** and **uncoating** during which virus introduces its genome into the host cell. There are many different strategies of entry amongst viruses and many specific virus structures adapted to the host cell. When the virion or the genome enters the cell, a virus loses the virion for a short period called the eclipse. The eclipse is the time between disassembly of the virion and assembly of new progeny virions. **Synthesis of viral proteins** and **nucleic acids** marks the beginning of virus replication. Synthesis of viral proteins and enzymes helps to mobilize the host cell's resources and permits efficient expression of the viral genome. Synthesis of viral structural proteins usually overlaps with the synthesis of viral nucleic acids. The new virion building material accumulates within the cell late in the infection. When there are plenty of replicated genomes and structural proteins available, **assembly** and **maturation** of new virions starts. The assembly of progeny from component parts is the defining feature of viruses. The last step is **liberation** of assembled progeny virions from the host cell. This process is variable depending on the host cell type and the virion structure (Adams 1959, Voyles 2002).

As the scope of this study is the double-stranded (ds)DNA bacteriophage, PRD1, and the mechanisms of packaging and lysis, this literature review will concentrate on these two steps in the life cycle. Mainly dsDNA bacteriophages will be discussed, as these viruses have been very successful model systems to study virus packaging and release.

2.1. Capsid assembly

Viral capsids are formed from multiple copies of one or several capsid proteins. This strategy is used in order to maximize genome coding capacity. Francis Crick and James Watson in 1956 proposed that coding of larger capsid proteins requires more space in the genome and this consequently leads to a need for bigger capsids.

Virion assembly can be considered simple or complex. Viruses utilising simple pathway assemble in a few steps, involving direct coat protein interactions with nucleic acid, while complex pathway viruses are assembled in at least two major phases: assembly of the precursor particle or procapsid followed by the maturation process (Luo 2008). Maturation usually involves packaging of viral nucleic acid into the procapsid and stabilizing the capsid with additional structures. Maturation is often associated with conformational changes in the capsid and its expansion (Dokland 1999, Jardine & Anderson 2006, Voyles 2002). Every phage has its own specific assembly pathway. Initiation depends on the formation of a small assembly of viral proteins that serves as a nucleation centre (Voyles 2002). Interactions

between the components of the nucleation centre cause conformational changes creating binding sites for the addition of new components. The components added to the growing structure are sub-assemblies of the capsid protein, such as pentamers or hexamers, rather than monomers (Luo 2008). Addition of new subunits form new binding sites and assembly continues according to the specific pathway. However, there is evidence of reversible of procapsid assembly in P22 (Parent et al., 2007). This can be explained by weak interactions between subunits, which were also observed in HK97, HBV, and other viruses (Ross et al., 2006, Ceres et al., 2004, Zlotnik 2007). The ability of the coat protein molecules of simple viruses to interact with each other, as well as the ability of the coat protein to interact with the genome, and the energy needed for these processes is intrinsic to their structures (Voyles 2002). Most of the viral genomes have specific sequences carrying encapsidation signals, which also serve as origins of assembly in simple viruses (OAS) (Voyles 2002). From energetic perspective the assembled structure is at a local energy minimum compared to free subunits, so the whole process is driven by thermodynamics (Caspar & Klug 1962).

2.1.1. Co-assembly

Co-assembly or co-condensation is a pathway utilised by simple ssRNA filamentous and icosahedral viruses where genome encapsidation and capsid formation occur simultaneously. The best-described example is TMV (tobacco mosaic virus) (Klug 1999, Navaratnarajah et al., 2008). Helical capsids, such as TMV, are built from identical coat protein subunits assembled around a single nucleic acid molecule, with possibly some minor coat proteins at the ends. Assembly of simple icosahedral viruses such as MS2 starts with binding of the coat protein dimer to a stem-loop structure within the viral ssRNA genome, which serves as a trigger for assembly by adding subsequent coat protein dimers (Stockley 2008). Another variation of co-condensation is pre-condensation, where the genome is first condensed by special proteins to form a compact nucleoprotein complex, which is subsequently covered by a protein shell. For example, the papovavirus circular dsDNA genome is first condensed by host cell histones. Viral capsomer complex recognises an encapsidation site located in nucleosome-free region. Binding of a capsomer to the genome acts as the nucleation signal and triggers polymerisation of capsomers around the genome while displacing the histones from the viral genome (Garcea & Liddington 1997).

2.1.2. Assembly of the preformed procapsids

Complex viruses, such as the tailed phages have branched assembly pathways. The head, tail and tail fibres are assembled separately and eventually joined together to form an infectious phage (Duda 2008). Formation of the head is a complicated task, as initially an empty spherical particle has to be assembled. This usually requires some help in the form of scaffolding proteins (Dokland 1999, Voyles 2002, Luo 2008). Scaffolding proteins are required for the correct assembly of the procapsid but are subsequently removed from it (King et al. 1980, Casjens & Hendrix 1988, Dokland 1999). The removal of scaffolding proteins can be done simultaneously with packaging or prior to it usually through cleavage by a virus-encoded protease. Alternatively, the scaffolding protein can be recycled and used for assembly of new particles as in phage P22 (King & Casjens 1974). Scaffolding proteins can

either be internal (as in P22, λ, herpes viruses), external icosahedrally ordered (e.g. phages P4 and ϕX174) or form a prolate core (as in T4) (reviewed in Dokland 1999).

Capsid assembly starts from a formation of an initiation complex often containing a 12-fold symmetric portal (connector) onto which the procapsid is assembled with the help of scaffolding proteins (Duda 2008). In bacteriophage P22, the scaffolding protein has been shown to interact with both the portal and the major capsid protein. In scaffolding mutants of P22, the portal is not incorporated in the capsid. In P22, HK97 and also HSV-1, the procapsid can be assembled with the help of scaffolding protein (or domain) without a portal, but these procapsids cannot be subsequently packaged. The portal protein is important for determination of correct size and shape in SPP1 (Dröge et al., 2000) and ϕ29 (Guo et al., 1991). Without a portal, aberrant particles are produced even in the presence of the scaffolding protein. However, it has been proposed for phage T7, that the capsid assembly is terminated by the addition of the connector and core complexes are incorporated (Cerritelli & Studier 1996). In addition to portals, scaffolding proteins are also important in the determination of the correct capsid size. Capsid proteins can often assemble in several different ways by altering the relative subunit positions but having similar local bonding interactions. In this way, a variety of alternative morphological forms can be produced, as in P22 and P2 scaffolding mutants (Thuman-Commike et al., 1998, Wang et al., 2006). Phage ϕX174 has both internal and external scaffolding proteins. Binding of the internal scaffolding protein to the coat protein causes conformational changes converting it into assembly-competent form. The binding of the spike complex and the external scaffolding protein then follows. The external scaffolding protein in turn mediates contacts between the pentamers of the coat protein and also between spike and coat protein, allowing formation of the icosahedron (Dokland et al., 1999, Voyles 2002, Dokland 1999). In bacteriophage HK97, the capsid assembles the correct size and shape without a separate scaffolding protein. The delta domain of the HK97 capsid protein serves as the scaffolding protein, mediating assembly, and is subsequently cleaved and removed from the particle by a viral protease (Duda 1995a, b).

2.1.3. DNA Packaging into preformed procapsids

The next task after building an empty procapsid is filling it with the viral genome. The best-known models relevant to this study are those of dsDNA bacteriophages and herpes viruses (Catalano 2005). These viruses fill their procapsids with DNA by the aid of packaging enzymes in an energy-dependent manner (Fig. 2). The packaging equipment and pathway they utilise depends on the genome replication type. Only few phages have "packaging ready" unit length genomes (ϕ29, PRD1 and P2) (Jardine & Anderson 2006). ϕ29 and PRD1 have genome terminal proteins covalently attached to the 5' ends of their genomes similarly to adenoviruses. The DNA-terminal protein complex is the packaging substrate (Guo et al., 1987a). Other phages produce long linear concatemeric DNA molecules of multiple genome lengths, which have to be cut into units appropriate for packaging (λ, P22, T3, T7 and branched T4) (Catalano 2005). In many cases, the DNA packaged into the procapsid is longer than one genome including the terminal redundancy (T7, P22, SPP1 and T4) or excised surrounding host DNA (in the case of phage genome integration into the host (Mu)) (Jardine & Anderson 2006, Rao & Feiss 2008, Catalano 2005). Quite a lot of

information about packaging has been obtained from *in vitro* packaging systems, which have been developed for many phages (e.g. ϕ29, λ, P22, T4, SPP1) during the last twenty years. While these systems differ in methodology, complexity and efficiency, they have been very helpful in determining the major players in the packaging process.

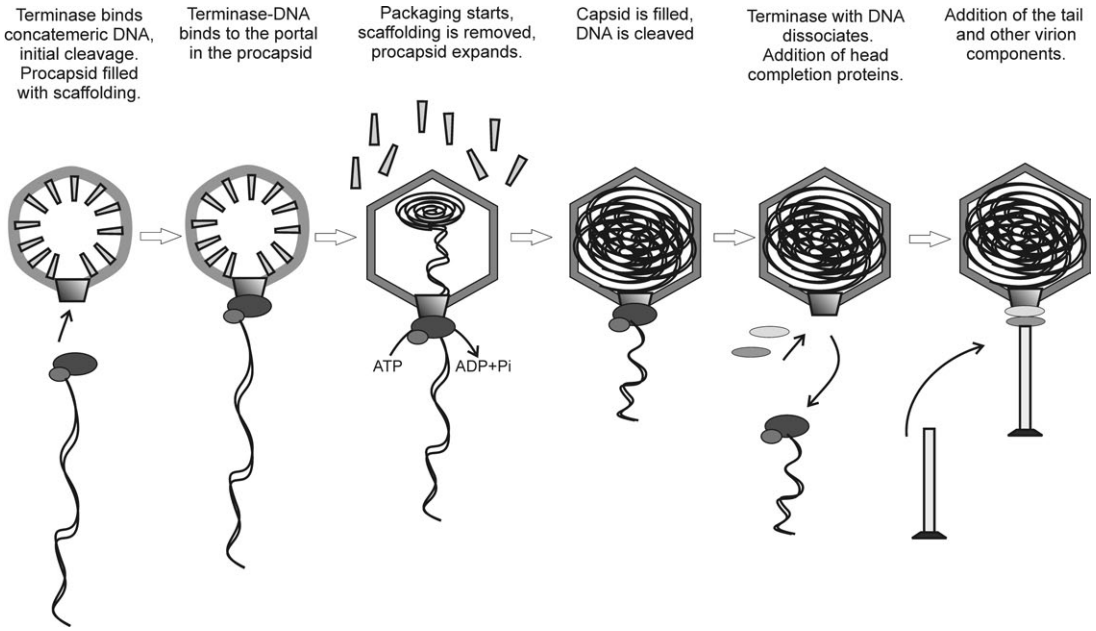

Figure 2. DNA packaging model for dsDNA bacteriophages.

2.1.3.1 Packaging enzymes

Depending on their DNA substrate phages employ different packaging enzymes. Phages with unit length genomes have packaging ATPases, and other phages that need to cut the DNA concatemer have 'terminases' - packaging ATPases with endonuclease activity. Essentially all terminases have two subunits, a large and a small one, that perform different tasks. In phage λ a functioning heterotrimer has been proposed to consist of two small and one large subunit (Maluf et al., 2005). The small subunit recognises and binds to the substrate DNA and positions the large subunit for DNA cleavage. Many small ATPase subunits have been predicted to have a helix-turn-helix motif within the amino (N)-terminus which is presumably involved in DNA recognition (Rao & Feiss 2008). The carboxyl (C)-terminus of phage λ small subunit has been shown to interact with the large subunit. Stimulation of the ATPase activity of the large subunit by the small one has been shown for many phages *in vitro* (Rao & Feiss 2008, Catalano 2005). The large ATPase subunit has endonuclease and ATPase activity and also binds to the procapsid (Jardine & Anderson 2006, Moore & Prevelige. 2002, Fujisawa et al., 1997, Guo & Lee 2007, Rao & Feiss 2008). The large subunits of packaging enzymes exhibit conserved sequence motifs of ATPases: Walker A and Walker B (Mitchell et al. 2002) needed for binding of ATP and Mg^{2+} (Walker et al., 1982) Some also have a C motif downstream of their Walker B motif. C motif is common for helicases, it couples the ATPase activity with movement of DNA (Rao & Feiss 2008). All well characterised large terminase proteins (of T4, λ, T3, SPP1) have an endonuclease

activity, which generates genome ends for packaging. This activity has been shown to reside in the C-terminal domain of the large subunits of T4 and λ terminases (Rao & Feiss 2008). The large subunits of T4 and λ terminases were also shown to have a DNA binding activity (Alam & Rao 2008, Ortega et al., 2007). The concatemeric DNA of *cos* (λ) and *pac* (T4, P22, SPP1, P1) phages is first cut at or near a specific site and subsequently produced DNA end is inserted into the procapsid to initiate packaging. A second cut is made after the genome is packaged. Whether the cut is specific or not, it is tightly linked to the filling of the capsid, which can mean packaging of either one genome length (λ) or 102-110 % of genome length (*pac* phages) due to headful packaging (Rao & Feiss 2008, Guo & Lee 2007).

There are several putative mechanisms to explain the cut in the headful packaging. The trigger of this cut can be a conformational change in the portal, which acts as a sensor. Alternatively, the terminase cannot cut the fast moving DNA, until it stalls when the head is full. Packaging ATPases are recycled (except for PRD1), after the packaging they are dismounted from the portal and used for packaging of other procapsids.

ϕ29 has a unique, small 174 nt packaging (p)RNA, which oligomerizes into a ring structure around the portal. Packaging RNA is required for docking of the large terminase subunit onto the portal and for stimulation of ATPase activity (Rao & Feiss 2008). pRNA is dismounted from the portal after packaging. Another peculiarity about ϕ29 is that the genome terminal protein, which is necessary for replication, is also necessary for packaging and it is thought to be analogous to the small terminase subunits of other phages (Guo et al., 1987a).

Viral packaging ATPases have been difficult to purify in an active soluble form (Huang & Guo 2003), and the only atomic structure available is for a non-functional mutant of T4 packaging ATPase domain (Sun et al., 2007).

2.1.3.2 Portals

Packaging takes place at one specific vertex of the icosahedral procapsid. In tailed phages and HSV-1, DNA is translocated through a cone-shaped structure, the portal (or connector for tailed phages), which is located at a unique five-fold vertex. The portal is a homo-dodecamer with a central channel large enough for passage of dsDNA. Some recombinant portal proteins have been reported to have different number of subunits, but all the portals analysed within the capsids structure are dodecamers (Rao & Feiss 2008). A narrow part of the portal protrudes outside the procapsid and provides the binding surface for the DNA translocation motor - terminase or packaging ATPase. X-ray structures available for ϕ29 and SPP1 portals (Simpson et al., 2000, Lebedev et al., 2007) show striking structural similarities to each other despite negligible sequence identity. For tailed phages the connector is the place for tail binding after packaging has been completed. In addition to their function in assembly and packaging, portals have been proposed to have a stimulating effect on large ATPase subunits in SPP1 and T4 (Oliveira et al., 2006, Bauman & Black 2003). In P22 it has been suggested that a conformational change of the portal causes a signal for DNA cleavage (Lander et al., 2006). Similar activities such as sensing of headful packaging and triggering the terminase cut have been assigned for the SPP1 portal (Orlova et al., 1999).

2.1.3.3 Accessory elements

After the genome is packaged and the ATPase is dismounted, the procapsid has to be sealed to withstand the internal pressure created by DNA confined against enormous

electrostatic repulsive forces within the limited space of the procapsid. Tailed phages have been shown to have 'neck' proteins that fulfil this purpose. They exhibit 6- or 12-fold symmetry, which matches the portal structure. Two such proteins are found in λ and SPP1 and three are found in T4 and P22 (Lurz et al., 2001, Catalano 2005). The neck proteins also provide the binding site for the tail. In some cases, host cell proteins are needed for packaging and in particular DNA bending. The *E. coli* protein integration host factor (IHF) has been reported to bind λ DNA cooperatively with the small terminase subunit and to bend viral genome. IHF has been suggested to promote the formation of an active DNA translocation complex essential for DNA cleavage and translocation (Maluf et al., 2005).

2.1.3.4 Forces in packaging

Single-molecule studies have shown that phage packaging motors generate an enormous force to package DNA. Forces up to 60 pN have been measured for ϕ29, λ and T4. Viral packaging motors are amongst the strongest biological motors known (Smith et al., 2001, Fuller et al., 2007a, b, c). DNA packaging is an energy-consuming task; the average ATP consumption is 1 molecule per 2 bp packaged in ϕ29 and T3 (Guo et al., 1987a, Morita et al., 1993). The rate of DNA translocation seems to correlate with genome size. The 20 kbp genome of ϕ29 is packaged at a rate of 100-150 bp/s, the 170 kbp T4 genome at ~ 700 bp/s, and the 48.5 kbp of λ at ~ 600 bp/s. This variation in rate can be an evolutionary adaptation in order to complete the infection cycle in time (Fuller et al., 2007a). It has been reported that the packaging rate decreases as the internal pressure in the capsid increases. In ϕ29 the packaging rate drops to zero when 100 % of genome is packaged (Smith et al., 2001), and in λ the three-fold drop is seen as 90 % is packaged (Fuller et al., 2007c).

Since 1978 there have been suggestions, that a symmetry mismatch between the hexameric or dodecameric portal and the five-fold vertex might cause rotation (Hendrix 1978), however so far this has not been proven experimentally. All the packaging mechanisms proposed could be categorized into two major groups: i) rotary models and ii) linear models (Rao & Feiss 2008). The rotary models that have been described include, nut and bolt (Hendrix 1978), DNA gripping (Guasch et al., 2002), molecular lever (Lebedev et al., 2007), and compression-relaxation (Simpson et al., 2000). These models propose that the rotation of the portal is coupled to the linear movement of the DNA. However, most of these models lack sufficient experimental evidence and recent single molecule investigation indicated that the portal does not rotate (Hugel et al., 2007). The linear models propose that linear DNA movement is achieved not by rotation, but by the linear motion of the terminase domains. In this group supercoiling (Black & Silverman 1978) and inchworm (Draper & Rao 2007) models have been described. However, more structural information on terminases will be required to determine which model(s) is the right one(s).

2.1.4. Capsid maturation

It is common for capsids of dsDNA bacteriophages to undergo structural changes upon packaging (Earnshaw & Casjens 1980, Jardine & Anderson 2006, Casjens 2008). It is still not clear if the removal of the scaffolding and capsid expansion precede and enable packaging or if they are triggered by it. In phages λ and T3, procapsid expansion occurs when about 30 % of DNA is packaged (Fuller et al., 2007c, Fujisawa et al., 1987), but in T4, expansion and packaging are not coupled (Rao & Black 1985). Capsid expansion is

irreversible, increases internal volume by 50-100 % and makes the capsid more stable (Rao & Feiss 2008). Maturation involves changes in the position of capsid proteins, conformational changes in subunits and even refolding of parts of the major capsid protein. In P22 surprisingly large holes in the capsid, necessary for removal of the scaffolding, are partially sealed during maturation process (Prasad et al., 1993). Refolding of parts of the capsid protein has been reported for P22, HK97 and HSV-1 (Jiang et al., 2003, Conway et al., 2001, Steven et al., 2005). Wang et al., (2003) have proposed that cleavage of the capsid protein enables the expansion of the procapsid, and that DNA packaging is needed for full maturation in phage P4. There are two models describing how the capsid expansion is triggered. The first: suggests a wave of structural changes arising from one point (portal) in the capsid caused by DNA (Prasad et al., 1993). The second suggests triggering the changes from the multiple sites in the capsid due to the interactions between packaged DNA and the capsid proteins (Conway et al., 2001).

2.2. Bacteriophage release

After progeny phages are packaged, matured and tails are fully assembled and attached they have to leave the exhausted host cell and infect new hosts. Bacteriophages use two basic strategies to escape their host (Young 1992, Young & Wang 2006). Filamentous phages like M13 encode a sophisticated secretion apparatus through which they and are continuously extruded from the host cell without killing it (Russel & Model 2006). In contrast, non-filamentous phages abruptly terminate their vegetative cycle by lysing the host. In general, lytic bacteriophages encode factors that weaken or destroy the bacterial cell wall (Young 1992, Young et al., 2000, Young & Wang 2006).

Host cell lysis has evolved to be a tightly controlled event, since the timing of lysis is critical for a phage to maximize its fitness (Wang 2006). On one hand, if lysis would occur too early in the infection cycle it would not be profitable, as too few progeny virions would be assembled. Delay of lysis, on the other hand, would delay progeny virions from infecting new hosts. These two opposing forces have been balanced during the course of evolution creating sophisticated lysis mechanisms (Wang et al., 1996, Young 2002, Young & Wang, 2006).

Lytic bacteriophages can be roughly divided into two classes, large and small, based upon genome size. Most large bacteriophages contain dsDNA genomes and encode at least two proteins to elicit host lysis. Small bacteriophages with ssRNA or ssDNA genomes encode a single lysis protein (Bernhardt et al., 2002).

2.2.1. Holin-endolysin system

All known dsDNA bacteriophages produce a soluble muralytic enzyme known as endolysin (Young et al., 2000). Most endolysins, do not have a secretory signal sequence so they require a membrane protein to gain access to the periplasm and the target peptidoglycan (Young et al., 2000). This small membrane protein is called holin, as it makes holes in the cytoplasmic membrane (CM) (Young & Bläsi 1995, Young & Wang, 2006) (Fig. 3). Holin functions as a timer for lysis (Wang et al., 2000). Endolysins with intrinsic export signals, designated SAR (signal-(anchor)arrest-release) domains, confer the capacity to effect host lysis without holin function. In such cases holins have only a control function rather than a

membrane permeabilisation one (Xu et al., 2004, 2005). In other holin-endolysin systems a holin inhibitor can also be produced allowing to achieve a very precise timing of lysis. Many phages infecting Gram-negative hosts have two overlapping lysis genes encoding accessory lysis proteins, Rz and Rz1 to permeabilize the outer membrane (OM) (Summer et al., 2007). In many cases the holin and endolysin genes are located adjacent to each other and are often clustered with auxiliary lysis genes in a "lysis cassette" that may encode up to five proteins (Ziermann et al., 1994). Holins and endolysins do not interact specifically with each other, which makes them interchangeable even between phages infecting Gram-positive and Gram-negative hosts (Steiner et al.,1993). Exchange of holin-endolysin system for a one-gene lysis system is more challenging, but possible (Zheng et al., 2008). The predominance of the holin lysis system in phages with large genomes comes from greater genetic malleability of this system, as it provides better evolutionary fitness (Zheng et al., 2008).

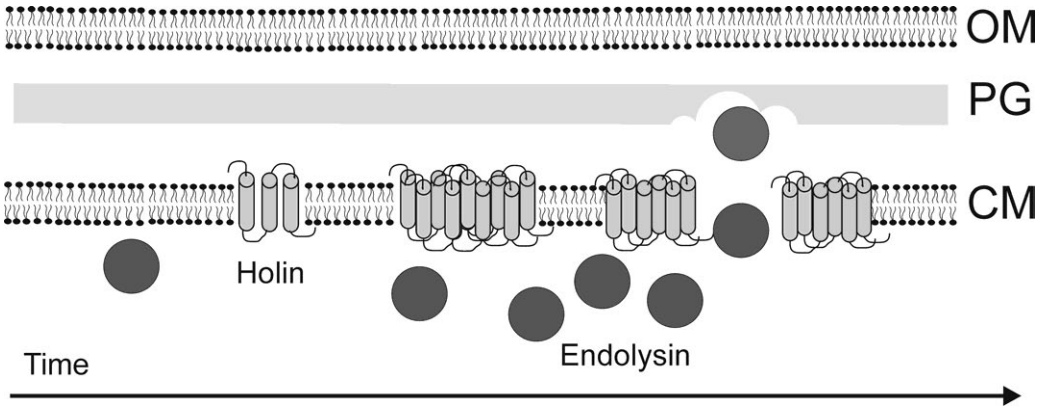

Figure3. Lysis model for large dsDNA bacteriophages.

2.2.1.1 Endolysins

The term endolysin is meant to distinguish lysis proteins from other muralytic enzymes, which are often found associated with the virion. The latter proteins glycosylases are needed for penetration of the murein sacculus of the cell during phage entry. At least four different muralytic activities have been found for endolysins: transglycosidases and lysosymes that affect the glycosidic bond linking amino-sugars in the cell wall; amidases and endopeptidases that affect the amide and peptide bonds of oligopeptide cross-linking chains (Young, 1992, 2002). Although many known endolysins are small globular proteins having one of the above listed enzymatic activities, large endolysins having two different activities have been reported for staphylococcal phages (Navarre et al., 1999).

Endolysins without intrinsic secretory signals accumulate in the cytoplasm during the infective cycle. It has been shown by Garret et al. using a phage λ holin mutant that if endolysin does not reach the peptidoglycan layer, cell culture mass and intracellular phage titers accumulate indefinitely. Endolysin activity also accumulates with no effect on cell growth or murein synthesis (Garret et al., 1981). Tendency of such cells to immediately lyse after treatment with chloroform, freezing and thawing or mechanical disruption implies dependence on a holin action (Reader & Siminovitch 1971b, Garret et al., 1981). In phages P1 and 21 endolysins are anchored to the membrane by their N-terminal SAR domain. These endolysins accumulate in the periplasm in an enzymaticaly inactive form. They become soluble and active when their SAR domains are detached from the membrane. This process can occur spontaneously if the CM is de-energized or in a controlled manner when holins depolarise the membrane (Park et al., 2007). In this case holins have been reported to form small holes for depolarization of the membrane and are consequently designated pinholins. However, endolysins (of phages infecting Gram-positive bacteria) that are independent of holin proteins in translocation through the CM, do exist (Sao-Jose et al., 2000). Phage fOg44 has an endolysin, lys44, with a cleavable N-terminal signal sequence, but it also encodes a holin, hol44. It is probable that in this system holin functions as an activator of the endolysin, since there are implications for lys44 to cross the membrane via the host *sec* machinery (Sao-Jose et al., 2000). Sequence comparisons revealed that the endolysins of some phages of Gram-negative bacteria also have N-terminal sequences, which could exploit the *sec* system (Young & Wang, 2006).

2.2.1.2 Holins

Holins compose one of the most diverse groups of functional homologs, with more than 50 unrelated gene families. Holins are usually short and hydrophobic proteins with a hydrophilic C-terminal domain. Holins can be grouped in at least three different classes according to their primary structures (Young & Wang, 2006). Class I consists of the phage λ(S)-like holins that are usually at least 95 aa residues long and harbour three potential transmembrane domains (TMD) and a periplasm located N-terminus. Class II holins are shorter, between 65-95 aa residues with two TMDs with both the N- and C- terminals residing in the cytoplasm, e.g. the holin of phage 21. Phage T4 has a class III holin. It is a long molecule with only one TMD and a relatively large periplasmic C-terminal domain and

the N-terminal end supposedly situated in the cell interior. Independent of the class, most of the tested holin genes complement λ holin *S* mutants (Wang et al., 2000).

The actual function of holins is not well understood due to the cloning and expression constrains on such lethal genes. The best studied holin is the S-protein of phage λ. Early studies have shown that the timing of lysis is dependent on the primary structure of holin, as different holin mutants exhibit different lysis timing and plaque formation phenotypes (Reader & Siminovitch 1971a,b, Young et al., 1979, Johanson-Boaz et al., 1994, Gründling et al., 2000a). Cross linking studies have suggested that the essential feature of the holin it's ability to form oligomers in the CM, as oligomerization in many lysis mutants is blocked at monomer, dimer or higher oligomer stages (Gründling et al., 2000b). It has been proposed (Wang et al., 2003) that holins form large two-dimensional aggregates in the membrane and at the time of triggering the lysis, TMDs form large protein-bounded lesions of about 12 nm in diameter, allowing passage of fully folded proteins. Recent work on λ holin (Savva et al., 2008), showed structures formed by the holin *in vitro* by electron microscopy (EM) and single particle analysis. λ holin purified in the presence of detergent forms rings of at least two size classes. The most common structure, composed of about 72 monomers has inner and outer diameters of 8.5 and 23 nm respectively. The holin complex is about 4 nm in height matching the span of the lipid bilayer. Holin mutant known to be oligomerization-deficient at the stage of dimer *in vivo* failed to form any of these structures. The unprecedented size of the central channel is consistent with the non-specific nature of the hole. This ring is big enough for passage of the λ endolysin and 15-45 such rings were estimated to assemble in the membrane during phage infection (Savva et al., 2008). It has been suggested that the action of λ holin is immediate – molecules accumulated in the membrane are inactive and have no effect on membrane integrity until (a few seconds before) lysis is triggered (Gründling et al., 2001), however experiments on other phages indicate otherwise (Gaidelyte et al., 2005).

2.2.1.3 Holin inhibitors

In addition to holin and endolysin, dsDNA phages often encode another lysis protein antiholin. In lambdoid phages antiholin is encoded by the same gene as holin. The translation initiation region of λ holin gene has two initiation codons and two Shine-Dalgarno sequences giving rise to two gene products. They are almost identical in sequence apart from extra residues at N-terminus. Similar holin gene structure is found in other phages with class II holins and designated a dual start motif (Bläsi & Young 1996, Barenboim et al., 1999). Antiholins are longer than holins by 2-3 N-terminal residues, one of which is often Arg or Lys, but these proteins have opposing functions. In λ the synthesis ratio of holin to antiholin is approximately 2:1. These two proteins are known to preferentially oligomerize in the membrane and in this way inhibit holin function (Gründling et al., 2000b). The functional difference of the two proteins in λ resides in one key residue of antiholin - positively charged Lys. The antiholin loses its ability of inhibiting holin when the membrane is de-energized by energy poisons or uncouplers, and seems to be converted into active holin (Bläsi et al., 1990). These findings have led to a model in which the N-terminal TMD1 in the antiholin is prevented from flipping up through the CM by the energized membrane (Graschopf & Bläsi 1999). Every antiholin molecule titres out one holin molecule, this process reduces the number of holin-holin dimers by 50 %. At the time lysis is triggered sufficient amount of holin-holin dimers causes an opening of the first hole, and the depolarisation of the

membrane allows the N-terminal TMD1 of antiholin in heterodimer to flip through the membrane becoming an active holin and permeabilising the membrane (Gründling et al., 2000c, Young 2002, Young & Wang, 2006). This is considered to be the "clock" regulation mechanism.

Antiholins encoded separately from the holins are also known. The best-studied independent antiholin is the RI protein from phage T4, which has no sequence similarity with the holin T of T4. RI is associated with the 'lysis inhibition' (LIN) phenomenon in the case of superinfection with T4. LIN depends only on RI, which has been shown to bind and efficiently inhibit the membrane embedded holin (Ramanculov & Young 2001). Antiholin of phage P1 is encoded by a gene adjacent but independent from holin, and is essential for the productive burst. In the absence of P1 antiholin lysis occurs catastrophically early, leaving no time for assembly of the progeny phages (Walker & Walker 1980). Phage P2 has a class I holin, but the antiholin appears to be a membrane protein with 4 TMDs and is predicted to have two basic cytoplasmic N-terminal aa residues reminiscent of the antiholins of the class II holins (Ziermann et al., 1994). However, the mechanism of function and specificity of P2 antiholin are not known.

2.2.1.4 Auxiliary lysis proteins Rz/Rz1

Phages of Gram-negative hosts have auxiliary lysis genes which in λ are designated *Rz/Rz1*. They are dispensable in laboratory conditions and their function is necessary only in the presence of millimolar concentrations of divalent cations, which stabilize the outer membrane and can contribute to the residual integrity of the cell envelope after endolysin action. Gene *Rz1* is fully embedded in *Rz*. Rz1 protein has a signal peptidase cleavage site and is predicted to be an outer membrane lipoprotein, while Rz is presumably a periplasmic protein (Young & Wang 2006). Not much is known about their function, but Rz is thought to be an endopeptidase, even though this activity has usually been associated with endolysins. *Rz/Rz1* type genes are found with three different types of endolysins, therefore there seems to be no dependence on the presence of *Rz/Rz1* and specific endolysin activity. In some phages like T1, *Rz/Rz1*-like genes have not been found, instead there are genes encoding "spanins". Spanins are outer membrane lipoproteins with a C-terminal domain capable of being incorporated into the inner membrane. Spanins are functional equivalents of λ Rz/Rz1 as T1 spanin has been shown to complement *Rz*$^{-}$*Rz1*$^{-}$ lysis defect (Summer et al., 2007).

2.2.2. Amurins

Bacteriophages with small genomes have severely limited coding capacity encode only one lysis protein. Moreover, the only lysis gene of these phages encompasses an alternative reading frame within another essential gene. In contrast to large bacteriophages, no lytic enzyme activity has ever been detected for small genome phages and attempts to identify phage-encoded endolysins have been unsuccessful (Bernhardt et al., 2002). Other alternatives for lysis would be the inhibition of peptidoglycan synthesis or induced host autolysis. Early on, it was observed that lysis induced by small phages shared many characteristics with penicillin-induced lysis, such as formation of membrane protrusions at sites of septation.

There three best-studied small phage lysis systems are those of ϕX174, MS2 and Qβ (reviewed in Young & Wang 2006 and Bernhardt et al., 2002). The E protein of ϕX174 has been shown to inhibit the function of an essential host integral membrane protein, MraY, that catalyses cell wall synthesis (Bernhardt et al., 2002). Lysis protein A_2 of phage Qβ, among many other functions, has been found to target another cellular protein, MurA, which is responsible for cell wall synthesis (Bernhardt et al., 2001a). It has been suggested that A_2 is a component of the progeny virion (Bernhardt et al., 2001b). Incorporation of A_2 in virions could provide a mechanism for regulation, as the accumulation of lysis protein and assembly of progeny virions would be coupled (Bernhardt et al., 2002). Bacteriophage MS2 has a lysis gene L encoded within the coat protein gene. The exact function of the gene L product is not known, but it has been observed to cause lesions in host cells and also trigger autolysis (Walderich & Höltje 1989, Walderich et al., 1988). It is possible that coupling of lysis and coat protein production ensures a fixed ratio of proteins and provides the means to coordinate timing of lysis (Bernhardt et al., 2002).

As this type of lysis does not destroy any existing structures but only inhibit the cell wall synthesis, a continued cell growth in required. Based on the discovered activity of small phage lysis proteins, the term *amurin* was introduced to indicate their negative effect on murein synthesis.

3. Bacteriophage PRD1

3.1. Structure

PRD1 is an icosahedrally shaped, tailless bacteriophage which is a type member of the *Tectiviridae* family (Olsen et al., 1974, Bamford, 2005). PRD1 has a membrane residing underneath the protein capsid. PRD1 genome is a linear, 14927 bp long DNA molecule with terminal proteins covalently attached to 5' ends and inverted terminal repeats at both termini. The genome is replicated by a protein-primed mechanism similar to the one proposed for adenovirus and ϕ29 (Caldentey et al., 1992, 1993). The genome is surrounded by a lipid membrane, which is composed of half lipids and half of phage specific proteins. (Davis et al.,1982). The membrane is acquired from the host, however, its lipid composition is altered. Virion membrane is enriched in PG and depleted in PE compared to the host membrane (Muller & Cronan 1983). This effect was initially proposed to reflect the origin of viral membrane - the CM, which is enriched in PG (Muller & Cronan 1983). The PRD1 membrane is also asymmetric due to high membrane curvature imposed by the small viral particle (Laurinavičius et al., 2004). This observation could also explain the extrusion of PE from viral membrane. The membrane is transformed into a tube during infection, and it is used as an injection device to transport the viral DNA to the host cell (Bamford & Mindich 1982, Grahn et al., 2002). Membrane vesicle closely lines the inner surface of the capsid. PRD1 capsid surface is composed of 240 capsomers, which are trimers of major capsid protein P3. Capsomers are arranged into an isometric capsid of pseudo T=25 icosahedral symmetry, as is also seen in adenovirus (Butcher et al 1995, San Martin et al., 2001, Abrescia et al., 2004). A minor capsid protein P30 running along the edge of the facets is required as a 'glue' and as a 'tape measure' protein. P30 is an extremely extended protein, two copies of which stretch out

from one five-fold vertex to another, determining the size of the capsid (Rydman et al., 2001, Abrescia et al., 2004). P31 pentamers occupy the vertices and form a base of the vertex complex, which is composed of the spike protein P5 and the receptor binding protein P2 (Rydman et al., 1999) (Fig. 4). Below the vertex complex resides protein P16, which connects the vertex complex to the membrane (Abrescia et al., 2004, Jaatinen et al., 2004).

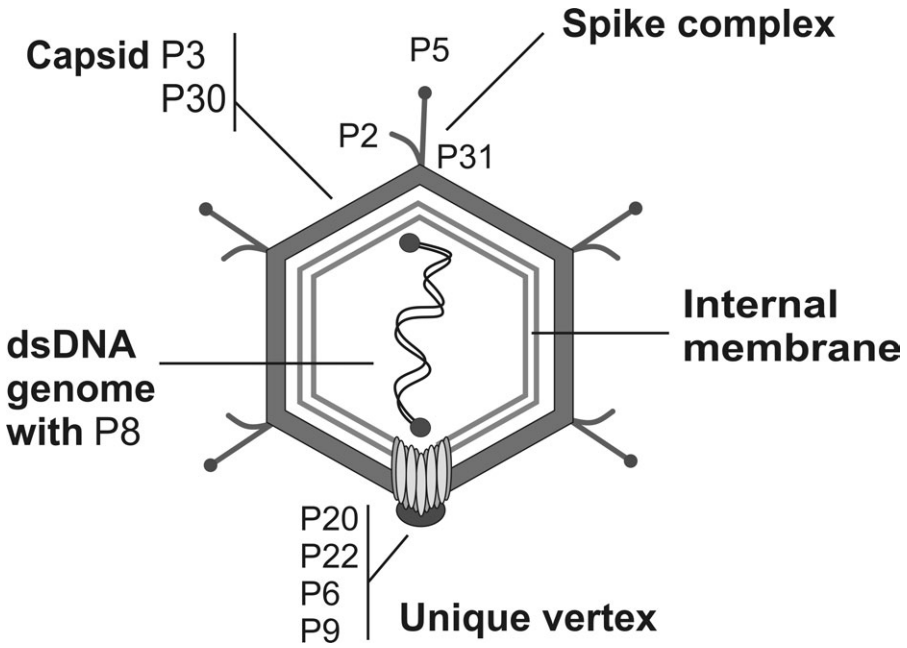

Figure 4. Schematic representation of the structure of PRD1. The exact arrangement of the unique vertex is not known.

3.2. Life cycle

PRD1 is known to infect a broad range of gram-negtive bacteria (e.g. *E. coli* and *S. enterica*, containing conjugative multi drug resistance plasmids of the IncP-, IncN- or IncW-type. Plasmid functions are dispensable for phage life cycle after the genome has entered the cell. The best-studied plasmid of the IncP type is RP4, which encodes the phage receptor, a cell surface complex. About 11 plasmid encoded genes belonging to the so called 'mating pair formation' (Mpf) system are necessary for PRD1 binding and entry (Grahn et al., 1997). The RP4 Mpf components were proposed to form a channel connecting CM and outer membrane (OM), facilitating DNA transfer into the host cell (Grahn et al., 2000). A single phage protein, P2, is responsible for the initial reversible binding to the host. The following irreversible binding step is mediated by a still unknown protein or mechanism (Grahn et al.,1999, 2002a). In addition to vertex complex proteins (P2, P5, P31) that are all needed for adsorption, proteins P11, P14, P18 and P32 are involved in DNA delivery. PRD1 mutants lacking P14, P18 or P32 proteins bind irreversibly to cells but are not infectious, as they are unable to form a membrane tube structure (Grahn et al., 2002). The genome has to cross the three layers of cell wall: the OM, the peptidoglycan layer and the CM. Penetration of the OM is dependent on P11, which makes way for P7, a peptidoglycan digesting enzyme (Grahn et

al., 2002a, Rydman & Bamford 2000). When peptidoglycan is digested, the phage lipid membrane acts as an injection device in DNA delivery (Grahn et al., 2002).

PRD1 initiates DNA replication from both ends of the genome by a protein-primed mechanism. DNA replication starts with a formation of covalent bond between the primer protein P8 and the 5' terminal nucleotide in a reaction catalysed by phage DNA polymerase P1 (Bamford et al., 1983, Savilahti et al., 1989). Subsequent to initiation, elongation of the initiation complex by the same polymerase takes place in a processive manner, resulting in the formation of full-lenth daughter DNA molecules (Savilahti et al., 1991).

At about 15 min post infection (p.i.) the major capsid protein P3, and the spike proteins appear in soluble form in the host cell cytosol, whereas the phage-encoded membrane proteins are delivered to the host cell CM. The correct folding of the soluble PRD1 proteins and the assembly of a number of viral membrane proteins are dependent on the host GroEL/ES chaperonins (Hänninen et al., 1997b). PRD1 proteins P10, P17 and possibly P33 are involved in capsid assembly but are not found in the mature virion (Mindich et al., 1982b, Bamford et al., 2002). PRD1 assembly proceeds via formation of virus-specific lipid vesicles covered with a scaffolding protein P10 and a small amount of P3 (Rydman et al., 2001). These vesicles develop from virus-specific membrane rafts presumably in a manner analogous to the formation of clathrin-coated pits. These 'membrane scaffolds' are then coated with P3 and P30, while P10 is displaced (Rydman et al., 2001). As a result, empty membrane-containing PRD1 procapsids are formed. They differ from mature virions only by the absence of the packaging ATPase P9 and the DNA-P8 complex. Procapsids are then packaged with the phage DNA by the packaging ATPase P9, which stays attached to the virion (Strömsten et al., 2003). The infection cycle is ended by host cell lysis. PRD1, similarly to many other bacteriophages, uses a two component, holin-endolysin (P35-P15) lysis system (Rydman & Bamford 2003). For schematic representation of PRD1 life-cycle see Figure 5.

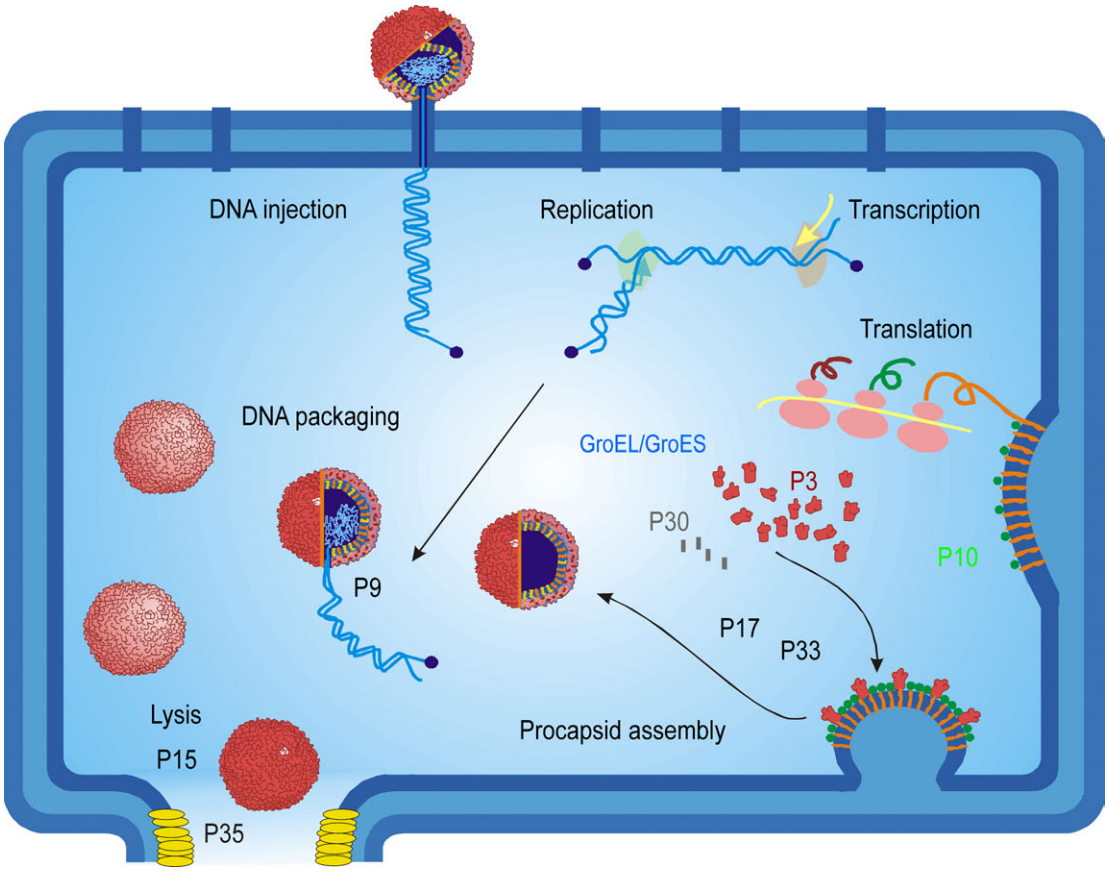

Figure 5. Life-cycle of PRD1. Modified from Grahn et al., 2006.

3.2.1. Packaging factors

PRD1 shares the packaging mechanism with other icosahedral dsDNA bacteriophages. The DNA is packaged into a preformed procapsid (Mindich et al., 1982) in an ATP-dependent manner (Strömsten et al., 2005). Analysis of PRD1 mutants has shown that at least three proteins are involved in packaging: packaging ATPase P9, and two small membrane proteins P20 and P22. The lack of P22 does allow some packaging, but the lack of P9 or P20 completely abolishes it (Strömsten et al., 2003). Unlike many other viral packaging ATPases, P9 is not recycled; it is a structural protein and stays attached to the mature virion (Mindich et al., 1982). According to sequence analysis, P9 is a classical ATPase having Walker A and Walker B motifs (Strömsten et al., 2005). A third specific motif found in P9 is shared with corresponding proteins in phage Bam35 and plasmid pBClin15 (Strömsten et al., 2005) and subsequently assigned as the P9/A32 motif. In further searches a similar motif was found in ATPases of several different viruses infecting bacterial, archaeal and eukaryotic species. All these icosahedral dsDNA viruses shared one common structural feature – an internal membrane (Strömsten et al., 2005).

The particularity of PRD1 is that there is no detectable procapsid expansion during packaging. Only the membrane vesicle inside the capsid adopts a more angular shape and increases its contacts with the major coat protein shell upon DNA packaging (Bucher at al., 1995). Another peculiarity is that there is no packaging-related removal of scaffolding proteins, which is often seen in tailed phages.

Packaging of PRD1 DNA proceeds through a unique vertex. The structure of the unique vertex is still unknown, but the components (P6, P9, P20 and P22) have been determined (Gowen et al., 2003, Strömsten et al., 2003). Minor capsid protein P6 and the small membrane protein P20 were found to be located at the unique vertex by immuno-electron microscopy (Gowen et al., 2003). The function of P6 was studied during the course of this thesis and will be described (I). Combining immuno-EM with further Western blotting analysis of the mutants revealed that packaging ATPase P9 and another small membrane protein, P22, are also located at the unique vertex (Strömsten et al., 2003). So far there are no indications of any protein structure in the capsid resembling the portal found in tailed bacteriophages. The major components necessary for PRD1 packaging were established by creating an *in vitro* packaging system for PRD1 (Strömsten et al., 2005), which was further studied and refined in this thesis (II).

3.2.2. Lysis factors

PRD1 is reported to have two proteins with muralytic activity. P7 is a peptidoglycan digesting transglycosylase enzyme, known to act during DNA entry (Rydman & Bamford 2000). P15 is a 1,4-β-*N*-acetylmuramidase associated with the liberation of progeny phages (Mindich et al., 1982, Caldentey et al., 1994). Analysis of phage nonsense mutants revealed that another phage gene, *XXXV*, is involved in host cell lysis. The product of this gene, protein P35, appeared to be a holin, most likely belonging to the class I lambdoid-type holins (Rydman & Bamford 2003). PRD1-infected cells were shown to lyse prematurely upon the addition of cyanide, giving support to the idea of a two-component lysis system (Rydman & Bamford 2003). The auxiliary lysis λ-type genes *Rz/Rz1* have also been found in PRD1 and have been designated *XXXVI* and *XXXVII*. Corresponding proteins P36 and P37 were shown to be interchangeable with the Rz/Rz1 proteins of bacteriophage λ (Krupovic et al., 2008). Further description of the holin-endolysin system of PRD1 will be provided in this study (III).

B. AIMS OF THE STUDY

PRD1 is a unique and well-studied phage. Its structure has already been solved to high resolution and a lot of data has been collected on the life-cycle of this virus, only the assembly of the virion and the host cell lysis were less characterised. The DNA packaging of PRD1 in principle is similar to that of dsDNA bacteriophages, but has some specific aspects, which make it worth studying. To investigate packaging process *in vitro*, a packaging system has been developed. This system was the first of its kind developed for a membrane-containing virus. However, many unanswered questions remained about the components and the conditions required for DNA packaging and also the principal mechanism of this process. The next step from packaging and maturation is virus release. PRD1 has been proposed to have a two-component lysis system like other large dsDNA bacteriophages; however, it was necessary to acquire experimental evidence about the acting components and the mechanism of this process. In the course of this study we were aiming to fill in these gaps and obtain clarity in the last steps of PRD1 infection. Our specific aims were:

- to determine the function of PRD1 packaging protein P6
- to purify all components of the *in vitro* packaging system: packaging ATPase, procapsids and DNA
- to optimise the *in vitro* packaging system using purified components and increase the packaging efficiency
- to find a method that allows to detect packaging separately from viral replication and to find the means to quantitate packaging products
- to determine the necessary components for the host cell lysis
- to establish the function of the holin and endolysin proteins

C. MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages used in this study are listed in Tables 1-3, respectively. Experimental procedures have been described in detail in the original publications and are summarised in Table 4. The references for published methods can be found from the articles.

Table 1. Bacterial strains used in this study.

Bacterial strain	Description	Relevant properties	Reference	Used in
E. coli K12				
DH5α	*supE44* Δ*lacU169* (ϕ80 *lac*Z ΔM15) *hsdR17 recA1 endA1 gyrA96 thi1 relA1*	cloning host	Sambook & Russell, 2001	I, II
HMS174	*recA1hsdR* Rf^r	cloning host	Campbell et al., 1978	I, III
HMS174(DE3)	*recA1hsdR* Rf^r	protein expression, host for gene cloning and complementation	Campbell et al., 1978	I, III
HMS174 (pcI857) (pTPH19)	*recA1 hsdR* Rf^R (pcI857)	expression strain for PRD1 P19	Pakula et al., 1993	I
HB101	*supE44 hsdS20*($r_B^-m_B^-$) *recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 mtl1*	Cloning host	Bolivar & Backman, 1979	I
DW720(pLM2)	DB178::Tc^R near *groEL*	complementation host	Hänninen et al., 1997	III
DW721(pLM2)	DW720 *groEL673*	complementation host *groEL* mutant,	Hänninen et al., 1997	III
DW719(pLM2)	DW720 *groES619*	*groES* mutant, complementation host	Hänninen et al., 1997	III
DW717(pLM2)	DW720 *groEL59*	*groEL* mutant, complementation host	Hänninen et al., 1997	III
DW716(pLM2)	DW720 *groEL44*	*groEL* mutant, complementation host	Hänninen et al., 1997	III
S. enterica LT2	serovar Typhimurium			
DS88	SL5676 ΔH2 H1-*i*::Tn*10*(Tc^s)(pLM2)	standard nonsuppressor host for PRD1	Bamford & Bamford 1990	I, II, III
DB7154(pLM2)	DB7100 *leuA414*(Am) *hisC527*(Am) *supD10*	suppressor host for *sus715, sus232* and *sus621* inserting Ser	Winston et al., 1979	I, II, III
DB7156(pLM2)	DB7100 *leuA414*(Am) *hisC527*(Am) *supF30*	suppressor host for *sus715* and *sus232* inserting Tyr	Winston et al., 1979	III
PSA(pLM2)	*sup*E	suppressor host for *sus715* and *sus232* inserting Gln	Mindich et al., 1982	III

Table 2. Plasmids used in this study.

Plasmid	Description	Reference	Used in
pLM2	Ap^r, Km^r, Tc^s, encodes PRD1 receptor	Mindich et al., 1976	I, II, III
pJB15	Ap^s, Km^s, Tc^r, encodes PRD1 receptor	Hänninen et al., 1997	I, II, III
pSU18	Low-copy-number cloning vector, p15A replicon, Cm^r	Bartolome et al., 1991	I, III
pET24	High-level expression vector, ColE1 replicon, Km^r	Novagen	III
pGZ119	Low-copy-number cloning vector, ColD replicon, Cm^r	Lessel et al., 1992	III
pET32	High-level expression vector, pBR322, Ap^r	La Vallie et al., 1993	III
pMG118	pSU18 + nt 2664-3141 from PRD1 genome (gene *XV*)	III	III
pGZ9	pET24 + nt 12984-13337 from PRD1 genome (gene *XXXV*)	III	III
pGZ15	pGZ119+nt 12984-13337 from PRD1 genome (gene *XXXV*)	III	III
pNS21	PRD1 gene *VI* with preceding Shine-Dalgarno sequence (nt 6769-7284) cloned into pSU18	I	I
pNS22	PRD1 gene *VI* (nt 6784-7284) cloned into pSU18	I	I
pJJ2	expression vector, Ap^R,	Ojala et al., 1993	I
pNS62	PRD1 gene *VI* (nt 6784-7284) cloned into pJJ2	I	I
(pcI857)	Encodes thermosensitive repressor for heat induced expression of proteins under the λ p_L promoter	Remaut et al., 1983	I
pPLH101	expression vector with λ P_L promoter,	Liljeström et al., 1988	I
pTPH19	PRD1 gene *XIX* cloned into pPLH101	Pakula et al., 1993	I
pMG60	expression vector ColE1 replicon, Ptac, Ap^r, T7 RBS	Strömsten et al., 2005	II
pGEX-6P-3	expression vector pMB1 replicon, Ptac, Ap^r	GE Healtcare	I
pNS96	wt P9 expression pMG60Δ(*Nde*I-*Hind*III)Ω(7624-8320), Ap^r	Strömsten et al., 2005	I
pNS98	His-tagged P9 expression pMG60Δ(*Nde*I-*Hind*III)Ω(7624-8320+His_6 C-term), Ap^r	II	II
pPP32	GST-tagged P9 expression pGEX-6P-3Δ(*Bam*HI-*Sal*I) Ω(7624-8320), Ap^r	II	II
pJB140	pBR322 *Pst*IΩPM2, Ap^r	Kivelä et al., 2008	II

Table 3. Bacteriophages used in this study.

Bacteriophage	Description	References	Used in
PRD1	wt	Olsen et al., 1974	I, II, III
sus715	nt 13006 G→A; Trp8(TGG)→Am(TAG) in gene *XXXV*	Rydman & Bamford 2003	III
sus232	amber mutant in gene *XV*	Mindich et al., 1976	III
sus1	amber mutation in gene *IX*, two missense mutations in gene *VI*	Mindich et al., 1982a	I, II
PRD1-1	lacz-α insertion (nt 6309)	Bamford & Bamford 2000	I, II
sus621	amber mutation in gene *VI*, two missense mutations in gene *VI*	I	I
Bam35	wt	Ravantti et al., 2003	II

Table 4. Methods used in this study.

Method	Used in
Growth and purification of PRD1 and mutants	I, II, III
Molecular cloning techniques	I, III
N-terminal amino acid sequencing	I, II
ATP measurements	III
Ion flux measurements and ΔΨ determination	III
Protein concentration determination	I, II
SDS polyacrylamide gel electrophoresis	I, II
Recombinant protein expression	I, II
Protein purification	I, II
Agarose gel electrophoresis and EtBr staining	I, II
Purification of viral DNA by phenol extraction and ethanol precipitation	I, II
in vivo complementation analysis of PRD1 mutants	I, II, III
in vitro DNA packaging	I, II
Transmission electron microscopy	I

D. RESULTS AND DISCUSSION

1. PRD1 DNA packaging

1.1. Unique vertex structure for packaging (I)

Previous immunolabeling experiments and analysis of PRD1 nonsence mutants (Gowen et al., 2003, Strömsten et al., 2003) suggested that the minor capsid protein P6 is an integral part of the unique vertex. Presence of P6 as well as of the packaging ATPase P9 in the unique vertex is dependent on two small membrane proteins P20 and P22. Mutants of P9, P20 and P22 are deficient in packaging. This suggested that P6 could also be involved in the packaging process. Such an idea was also in line with the fact that the location of gene *VI* in PRD1 genome is in front of the ATPase gene *IX*. It also overlaps with gene *X* coding for non-structural protein P10, which plays an important role in the assembly of PRD1, when a virus specific lipid vesicle is formed onto which the major capsid protein is assembled. PRD1 and other tectiviruses (Ravantti et al., 2003) are known to have the genes coding for proteins with related functions located close to each other in the genome. A P6 mutant could thus provide further insight to the function of P6 in the assembly and genome packaging of PRD1.

We have isolated a suppressor-sensitive mutant sus621 defective in PRD1 gene *VI*. The analysis of the mutant-infected cells and the purified mutant particles revealed that capsid assembly was not affected; the particles produced were of normal shape and size (I).

The estimated amount of particles produced in the sus621 mutant infection was comparable to that of wt PRD1. However the packaging process was significantly impaired, as about 95 % of particles produced were empty. The remaining 5 % of mutant particles were found to be packaged, mature and infective.

Detailed analysis of structural proteins in the P6-deficient mutant particles by SDS-PAGE and Western blotting revealed that no other protein was missing. Empty viral particles were found to contain some P9, which is not a component of wt empty particles. However, filled mutant particles contained less P9 than wt particles. Surprisingly, empty mutant particles contained a non-structural viral protein P19. P19 is known to bind DNA, but is neither a component of filled or empty wt PRD1 particles (Pakula et al., 1993).

Analysis of sus621 particles in sucrose gradients and those in EM of thin sections of mutant-infected cells revealed similar portions (~95:5 %) of filled and empty particles in both preparations. This suggests that filled particles are stable and there is no DNA leakage during purification. We further purified and carried out stability tests on the filled sus621 virions. Over a period of four weeks the specific infectivity of these virions remained the same (1×10^{11} pfu/mg protein). It was lower than for wt filled particles (1×10^{13} pfu/mg), but much higher than for the empty sus621 or empty wt particles (1×10^{10} and 5×10^{9} pfu/mg respectively).

However, it was difficult to distinguish empty and filled sus621 particles by negative stained EM (or in density gradients). Filled sus621 mutant particles seemed much more susceptible to stain penetration than wt particles. It has been shown (Luo et al., 1993) that in contrast to wt DNA-filled particles the interior of the packaging ATPase P9 mutant sus1 particles is accessible and the membrane can be removed by SDS treatment. We tried this technique on sus621 particles. The empty sus621 particles were found to behave similar to

sus1, they lost their inner membrane after SDS treatment, which shows that the presence of P9 does not protect the particle interior when P6 is missing.

The DNA packaged by P6 mutants was extracted and analysed. It appeared to be 15 kb long and gave a restriction map identical to that of wt PRD1 DNA. DNase treatment of virions during purification did not reduce the amount of PRD1 specific DNA, which suggests that the sus621 DNA was properly packaged.

As 95 % of mutant sus621 particles were empty, they were tested for *in vitro* packaging. Packaging was performed essentially as described previously (Strömsten et al., 2005). Sus621 particles were provided as a mutant infected cell extract and the missing P6 protein was also provided as an *E. coli* extract containing recombinant protein. Surprisingly, addition of the packaging ATPase P9 was necessary for packaging, even though P9 is expressed in wt amounts in sus621 infection, and should be present in the packaging reaction. In the reactions to which P9 was added 1 % *in vitro* packaging could be detected even in the absence of P6. Addition of P6 further increased packaging activity about 3 fold. However, packaging efficiency of sus621 particles was always less than 3 % of that obtained with sus1 particles (I).

Our study of the P6 mutant (I) confirmed the participation of this protein in the DNA packaging process of PRD1. However, its mechanism of action is not obvious. The packaging systems of icosahedral dsDNA phages contain proteins, which seal the capsid after DNA has been packaged. The possible function of P6 as a "plug" alone can be ruled out, because packaged sus621 mutant particles seem to be stable, mature and infective. Also, the function of P6 as a portal is unlikely, since the assembly of the mutant capsids was not affected and packaging, though inefficient, does occur. It is likely that P6 has a role in incorporating P9 into the unique vertex. The expression of P9 during sus621 infection is normal, but in the purified mutant particles we see significantly reduced amounts of P9 (~50 %). This correlates with the fact that in the *in vitro* packaging experiments no plaques were seen without addition of extra P9, even though P9 is incorporated into sus621 procapsids. Probably in the absence of P6, incorporation of P9 into the capsid is impaired or inefficient, or the incorporated P9 is not active. Presence of P9 and DNA binding protein P19 in the empty sus621 particles also suggest that those particles are products of unsuccessful packaging.

Wt procapsids and fully packaged mature particles differ only by the presence of P9 and the DNA-P8 complex. Stabilization of the packaged particle is most likely dependent on those two proteins, since no conformational changes in the capsid have been observed. Sealing of the capsid could be achieved by some kind of a conformational change in P9 or its interaction with P6. Also P20 and P22 as well as P8 (due to its hydrophobicity) could be involved in sealing the capsid at the membrane level. It is likely that P8 resides in a close proximity to the membrane, since the genome should be ready for the ejection.

1.2. A model for PRD1 DNA packaging (I)

Based on our findings and previous data we propose the following model for DNA packaging (Fig. 6). Procapsids containing the membrane are assembled while genomes with terminal protein are synthesised separately. Packaging is probably initiated by the binding of packaging ATPase P9 to the unit length PRD1 genome. Whether this occurs through binding to DNA or to covalently linked terminal protein is not clear. There is no evidence of a

packaging-specific sequence in the PRD1 genome, but the terminal protein is known to be necessary for packaging of PRD1 (see next chapter). We propose that the P9-DNA-P8 complex binds the empty procapsid with the help of P6, which in turn is connected to small membrane proteins P20 and P22. P20 is most likely the a membrane-spanning pore through which DNA is packaged into the membrane vesicle, since unlike lack of P20, lack of P22 allows some packaging (Mindich et al.,1982b). As the DNA is being packaged, it pushes the membrane against the capsid and reduces the distance between lipid head groups and capsid proteins (Butcher et al.,1995). The ATPase stays attached to the virion after the PRD1 genome packaging has been completed (Mindich 1982a). A conformational change or interaction between the packaging proteins, or the presence of the terminal protein P8, may act to seal the packaging vertex.

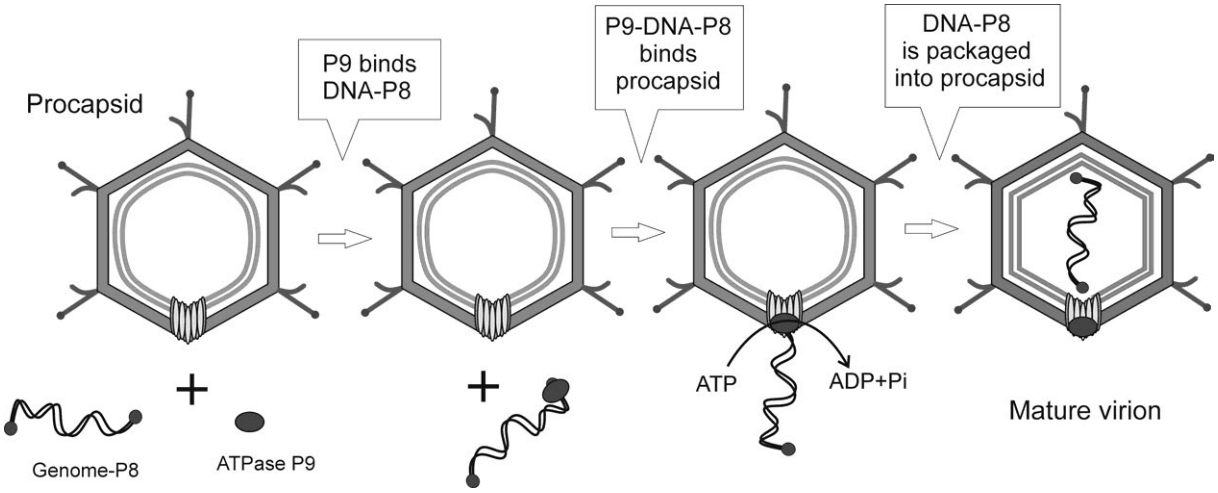

Figure 6. Packaging model for PRD1.

1.3. *In vitro* packaging of PRD1 (II)

One of the best ways to study packaging is to develop an *in vitro* packaging system (Fig. 7). Previously, such a system has been developed for PRD1 (Strömsten et al., 2005). However, this system had drawbacks. The efficiency of packaging system using the endogenous DNA (sus1 mutant genomes from cell extracts) (1 x10^9 pfu/ml reaction) was comparable to the best-developed systems (ϕ29, T4, lambda), however, in packaging of exogenous DNA (purified PRD1-1 genomes) the efficiency was quite low (5 x10^6 pfu/ml reaction) (see II for details). Apparently the exogenous DNA added to the system could not compete with the abundant, intact endogenous DNA already present in the system (a consequence of replication of the sus1 mutant genome). Another drawback of the system was that procapsids and packaging ATPase were provided as cell extracts, which did not allow elimination of the possibility that some additional unstructural viral or cellular components

might contribute to the packaging process. The most obvious solution to these problems was purification of procapsids and packaging ATPase, and optimisation of the new system.

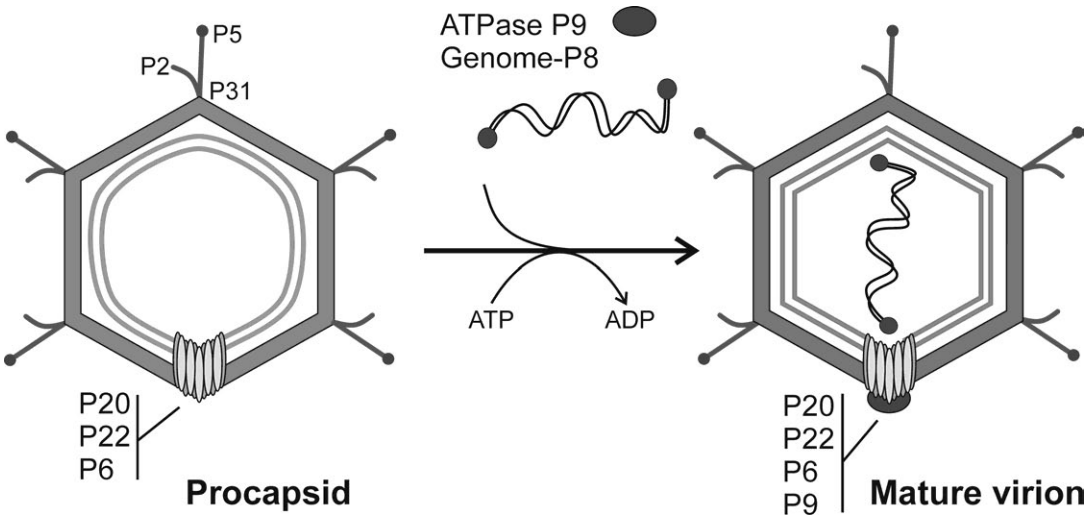

Figure 7. In vitro packaging system of PRD1.

Purification of procapsids using standard PRD1 purification protocols was not successful. Procapsids were very sensitive to NaCl and sucrose (II) i.e. to osmotic pressure. Consequently the particle concentration step using PEG-NaCl precipitation after cell lysis had to be replaced by harvesting of cells by centrifugation, followed by sonication. In order to reduce the DNA background in the procapsid preparation originating from free phage particles we introduced an additional washing step of harvested cells. We also optimised buffers, density gradients and concentrating techniques for purification. Our new purification protocol allowed us to produce packaging competent procapsids, which could also maintain relatively high packaging activity during storage (see II).

Our attempts to purify packaging ATPase P9 were not as successful. As wt P9 expression resulted in rapid precipitation of the protein we made two constructs for recombinant N or C- terminally tagged P9 expression. Both constructs gave reasonable amounts of soluble protein. However, when we tested these proteins in the *in vitro* packaging assay their enzymatic activity was significantly compromised. Further purification steps and addition of ATP or Mg^{2+} ions resulted in rapid precipitation of the proteins. We were unable to produce soluble and biologically active purified P9, which forced us to continue the usage of the cell extract. However, the extract contains no other viral proteins besides P9, and thus would not compromise our *in vitro* packaging system.

Optimisation of the *in vitro* packaging system using purified particles started with saturating the reaction with DNA and procapsids, respectively. Our results were in contrast with those previously obtained by Wong & Bryan (1978) about the weight ratio of DNA versus protein in the PRD1 virus particles, which was estimated to be 1:5. For optimal *in vitro* packaging we needed ~10 times more procapsids (1:54) than expected, suggesting that the procapsids are fragile or damaged during purification. This observation led us to test the

stability of the components of the packaging reaction (see II Fig. 4a). The PRD1 DNA appeared to be the most stable component in the packaging conditions used; however, procapsids lost about 20 % of packaging activity in 15 min at 37°C. The most drastic drop in packaging activity was observed in packaging ATPase P9 (95 %). This result is in line with our observations during P9 purification and could be explained by rapid precipitation of the protein.

As the previously published packaging system was our starting point for development of the new one, we used the same buffering conditions, which now needed to be tested and optimised. We removed salts and PEG from the reaction buffer one at the time and checked for the packaging efficiency. Removal of PEG or Mg^{2+} had a strong adverse effect on the packaging; however, the removal of NaCl significantly improved the efficiency. This was not surprising as we already observed that NaCl caused an adverse effect during particle purification. Similar observations have been made with phage λ (Gaussier et al., 2006). In our unique system with membrane containing procapsids it is probable that osmotic effects of NaCl and sucrose could compromise viral membrane integrity.

Our further challenge was to find an optimal pH and buffer composition for the packaging reaction, as all the major components of the reaction have their own optimal buffers and preferred conditions. The best packaging efficiency could be obtained at pH 7, however, previously used Tris-HCl was no longer the optimal buffer. Much better results were obtained with Bis-Tris buffer, which was chosen for further use (II).

Removal of endogenous DNA from the particle preparation and optimisation of the system improved the efficiency of exogenous DNA packaging 50 fold. We observed the same plaque counts on both suppressor and nonsuppressor hosts, which indicated that there is no detectable residual endogenous DNA in the procapsid preparation. Testing of the procapsid preparation for DNA also confirmed this finding. The levels of endogenous DNA packaging, however, could not be reached. It has to be noted, that the newly developed packaging reaction contains procapsids and DNA, which undergo rounds of purification and are exposed to potentially damaging physical and biochemical factors. In the previous packaging system endogenous DNA and procapsids were contained in conditions close to *in vivo*. It is also noteworthy that the exogenous PRD1-1 DNA used is 393 bp longer than the wt PRD1 and may not be packaged with the same efficiency as the wt DNA.

1.4. Applications of *in vitro* packaging system (II)

The existence of an *in vitro* packaging system free of endogenous DNA allowed us to address old question, namely whether the genome terminal protein is needed for packaging? We designed a competition assay, to compare different kinds of DNA molecules provided for the packaging reaction. Not surprisingly, PRD1 DNA without the terminal protein or other DNA molecules did not compete or interfere with packaging of the P8-DNA complex. Even though we cannot explain the function of PRD1 P8 in packaging at this point, our data indicated that the terminal protein is essential for packaging similarly as has been shown for phage ϕ29, which also contains terminal proteins covalently linked to the 5' ends of the genome (Grimes & Anderson 1989). We presume, that P8 has a recognition site for binding to packaging ATPase or procapsid.

We also determined the packaging rate. Very accurate rate and load measurements for phages ϕ29, T4 and λ have been done recently by Fuller et al., (2007a,b,c) using optical tweezers. The average packaging rates measured from 165 bp/s (ϕ29) to 700 bp/s (T4). Our preliminary experiments with PRD1 put us in the same range in terms of approximate rate of packaging (~350 bp/s). It has to be noted, that our measurements represent not just translocation of DNA, but also the preassembly of the packaging complex and capsid maturation. Preassembly experiments could not be carried out due to the instability of the packaging ATPase. The maturation step of the packaged procapsid is not detected at this point (see above) and is considered to be instantaneous. Experiments with optical tweezers would be needed to obtain precise packaging rates and forces and our improved packaging system brings this goal within the reasonable reach.

1.5. DNA translocation assay (II)

Not only was the purification of membrane containing procapsids challenging, but also genome terminal protein caused some difficulties. P8 is absolutely necessary for genome replication and the most widely used assay for packaging is scoring of plaques, which in turn appear only after a successful completion of life-cycle. Consequently we face a problem that the inability of packaged particles to replicate (e.g. damaged P8 during DNA purification) can give a misleading result. We needed a packaging assay, which would show the translocation of DNA and would not depend on any subsequent steps in the viral life cycle (infection, replication, lysis etc.). We used our previous knowledge about the distribution of the empty and full PRD1 particles in density gradients as separate bands (Bamford & Bamford 1991). We optimised the centrifugation conditions to increase the separation of full and empty particles obtained from *in vitro* packaging reactions. As after density centrifugation these particles were found in separate bands, we subsequently fractionated the gradients and analysed fractions for protein and DNA content and plaque formation. We could see the *in vitro* packaged particles as a separate gradient zone, and were also able to detect DNA and infectious particles in the same zone. As a result we obtained a new method for detecting DNA translocation into procapsids. According to our calculations by the DNA translocation assay we could detect one order of magnitude more particles being packaged in the same packaging reaction than detected by the plaque assay. This observation proves our concern, that plaque assay does not dissect the DNA packaging from viral replication and gives us underestimate of packaging efficiency.

2. Release of mature particles (III)

Like most bacteriophages, PRD1 uses a two-component lysis system to release mature virus particles. It was shown that premature lysis can be triggered by addition of energy-depleting agents like cyanide or dinitrophenol (Rydman & Bamford 2003). We employed different metabolic inhibitors to pinpoint the metabolic processes of the host cell involved in triggering of premature lysis. Addition of inhibitors to noninfected cells in control experiments showed, that arsenate and cyanide drastically affected the ATP content of the cells, but only cyanide depleted the membrane voltage. In experiments with PRD1 infected

cells arsenate was even more effective than cyanide in causing premature lysis. However, nonsuppressor cells infected with holin and endolysin mutants failed to lyse prematurely after the addition of arsenate or cyanide, indicating that both holin and endolysin proteins are needed for both the premature and the normal host cell lysis.

As the function of the holin is to permeabilize the CM for endolysin passage, it should also allow a nonspecific movement of ions and small molecules across the CM. We used the ion-selective electrodes to analyse holin functions. K^+ leakage from PRD1 infected cells was detected at 40 (± 2) min p.i. (error estimated from 3 or more independent experiments), which well precedes the normal lysis. No such leakage was seen in infection with holin deficient mutant or with noninfected cells. Membrane voltage (ΔΨ) during the infection was followed using a TPP^+-selective electrode, as the distribution of TPP^+ between the cell cytosol and medium is a ΔΨ-dependent process. Yet another holin dependent process was observed - a significant decrease in ΔΨ at about 55 (± 2) min p.i., which precedes the lysis by ~ 10 min.

The observed effects of metabolic inhibitors on the ΔΨ and intracellular ATP concentration of *S. enterica* cells, and their ability to cause premature lysis suggested that the lysis process is an ATP dependent event. ATP levels is PRD1 infected cells showed a rather sharp decrease of intracellular ATP after 30 (± 2) min p.i., which in turn was followed by K^+ leakage - the first sign of holin dependent CM permeability (at about 40 (± 2) min p.i.) indicating that enough holin molecules are incorporated into CM to allow the K^+ leakage. A second significant decrease in ATP was observed at about 60 (± 2) min p.i., and was instantly followed by a decrease in optical density of cell suspension. This second decrease can be explained by the leakage of ATP from the lysing cells, as the extracellular ATP level increases at about the same time.

Taking all those observations together it can be suggested that there is an ATP-dependent lysis control mechanism. It may be related to ATP-consuming DNA packaging, which in time coincides with observed drop in intracellular ATP level.

It was previously known that GroEL/GroES chaperonins assist in the insertion of PRD1 proteins into the virion membrane (Häninen et al., 1997a,b). The chaperonins were also known to solubilize λ holin and translocate some other membrane proteins to CM in a process inducible by low ATP (Deaton et al., 2004, Bochkareva et al., 1998). It is possible that chaperonins could be involved in the storage and delivery of holin molecules to CM. We tested the lysis of PRD1-infected temperature sensitive *groE* mutant cells. All mutant strains lysed normally at permissive temperatures, but did not show any signs of lysis at nonpermissive temperatures. Infected wt strains lyse normally at both temperatures.

2.1. Host cell lysis caused by recombinant proteins (III)

To enable specific assessment of the effects of the holin and endolysin proteins on the CM, the corresponding genes were cloned and expressed (see III for details). The functionality of the recombinant holin proteins was confirmed by a complementation assay. All previously observed effects on the infected cell physiology were tested with plasmid-borne holin and endolysin. K^+ leakage, decrease in accumulation of TPP^+ and stop of growth could be observed in cells expressing holin or both holin and endolysin genes after induction with IPTG. However, lysis occurred only in cells expressing both of these proteins. Cells expressing only endolysin, as well as control cells, did not leak K^+, or show a decrease in the

accumulation of TPP^+ and continued to grow. ATP measurements in cells carrying both lysis genes showed an increase in ATP level after induction followed by a sudden drop.

Taken all these observations together we can conclude that K^+ leakage and the decrease in $\Delta\Psi$ are holin dependent processes, but for the cell lysis an endolysin is also required. Cells carrying lysis genes were also tested for the premature lysis effect. After addition of arsenate, cells with both lysis proteins showed immediate signs of lysis, while cells containing only holin or endolysin showed a delay in or complete lack of lysis, respectively. This is another indication that both of the PRD1 lysis genes are necessary for lysis of the host cells in laboratory conditions.

2.2. Multi-step lysis (III)

We propose a following multi-step lysis model. Lysis is initiated quite early in the the PRD1 life-cycle by a drop in intracellular ATP concentration caused by the energy consuming process of DNA packaging. This in turn causes leakage of intracellular K^+, as the low ATP levels cause groE-associated holin molecules to incorporate into the CM. K^+ leakage leads to the dissipation of $\Delta\Psi$, which mediate the opening of endolysin-permeable holin pores, and the endolysin degrades the peptidoglycan, leading to the cell lysis.

E. CONCLUSIONS AND FUTURE PROSPECTS

This study concentrates on late steps of PRD1 life-cycle: DNA packaging and host cell lysis. The function of packaging efficiency factor was assigned to the unique vertex protein P6. The purification of the components of the *in vitro* packaging system revealed new insights into the mechanism of PRD1 DNA packaging. Dissection the packaging of endogenous and exogenous DNA was very important in understanding advantages and limitations of our *in vitro* system. The semi-purified system enabled us to answer critical questions regarding the need of the genome terminal protein P8 for packaging. We were also able to address the question of the rate of packaging, which was shown to be similar to the rates of several other virus packaging systems. The newly developed DNA translocation assay dissected packaging from viral replication. It demonstrated the real efficiency of the system and presented several new options to evaluate packaging *in vitro*. We were able to propose a plausible model for PRD1 packaging.

The study of PRD1 lysis revealed many new findings. It was the first time any physiological changes in infected cells well preceding lysis were observed. Our results contradict some previous works on phage lysis. However, our findings neatly describe the sequence of phage-dependent events leading to the lysis of the host cell.

The proposed dependence of lysis on intracellular ATP level may connect the DNA packaging and lysis steps of phage infection to a logical time-coordinated pathway.

The semi-purified *in vitro* packaging system could be applied to single-molecule studies, which in turn would give us even more insight into the mechanism and kinetics of DNA packaging. Possible further studies on the packaging ATPase would require a more resilient approach, but would be very useful as PRD1 P9-type ATPases are found in viruses infecting bacterial, archaeal and eukaryotic hosts. The future research on PRD1 packaging should answer the questions of interactions between packaging proteins and DNA as well as the sequence of events.

F. ACKNOWLEDGEMENTS

This work was performed in the Finnish Academy Centre of Excellence in Structural Virology/Virus Research (2000-2005 and 2006-2011), at the Institute of Biotechnology and Department of Biological and Environmental Sciences, at the University of Helsinki, under the supervision of Professor Dennis Bamford.

I wish to express my deepest gratitude to my supervisor Dennis for all these years of research and support, for taking me into his lab and giving me the opportunities I never dreamed about, for all I have learned here, for knowing, understanding, believing in and caring about his students, for the push I sometimes needed.

Warm thanks go to my second supervisor Professor Jaana Bamford for introducing me to the fascinating world of DNA and our fruitful collaborations.

Docent Mikko Frilander and Dr Roman Tuma are thanked for carefully reviewing the thesis manuscript and for their extremely helpful comments. Professors Kristiina Mäkinen and Kari Keinänen are thanked for their support and advice on my work as members of my follow-up group.

The Viikki Graduate School in Molecular Biosciences and especially former coordinator Eeva Sievi are thanked for making my studies enjoyable.

I would like to express my appreciation to all my co-authors Rimas, Nelli and Hanna, for fruitful collaborations. Rimas as my former supervisor is especially thanked for giving me the opportunity to go to Finland, which he might have regretted. I was very lucky to work with Nelli, one of the brightest people in the lab. Even if we had tough times with our project, I had someone to look up to. I want to thank Hanna for keeping an eye on me at difficult times.

I would like to acknowledge Riitta Tarkiainen, not just for excellent technical support, but for being the soul of the lab, the one person you can always count on for help, advice or just moral support.

Over the period of my Ph. D. studies I have met so many special people Janne, Sampo, Antti, Harri, Peter, Petra, Simonas, Vio, and many others. Thank you for all the great times, laughs and litres of coffee we have shared together. I am grateful to Antti for his honesty and friendship. Janne will be remembered for always keeping me on the edge. I thank Peter for his help and our endless discussions in the office.

I am grateful to Dr James Thompson for his time and will, good advice and more importantly support and encouragement during preparation of this thesis.

My dear friends Aušra and Andrii are especially thanked for many nice years we had, all parties and trips, you really made Finland more home for me.

And of course my love goes to my family, my parents Ramūta and Gintautas and my brother Marijus. I wouldn't be here without them, their love and support through the years.

Gabija Žiedaitė
Helsinki, October 2008

G. REFERENCES

Adams, M. H. (1959). Bacteriophages. Interscience Publishers, INC., New York.

Abrescia, N. G. A., Cockburn, J. J. B., Grimes, J. M., Sutton, G. C., Diprose, J. M., Butcher, S. J., Fuller, S D., San Martin, C. Burnett, R. M., Stuart, D. I., Bamford, D. H. & Bamford, J. K. H. (2004). Insights into assembly from structural analysis of bacteriophage PRD1. *Nature* **432,** 68-74.

Ackerman H. W.(2005). Bacteriophage classification. in Bacteriophages biology and applications. Kutter, E. & Sulakvelidze, A. eds. CRC Press.

Ackerman H. W.(2006). Classification of Bacteriophages. *in Bacteriophages.* R. Calendar ed., IIed.

Ackerman H. W.(2007). 5500 Phages examined in the electron microscope. *Archives of Virology* **152**, 227-243.

Agirrezabala, X., Martín-Benito, J., Valle, M., González, J. M., Valencia, A., Valpuesta, J. M. & Carrascosa, J. L. (2005). Structure of the connector of bacteriophage T7 at 8Å resolution: structural homologies of a basic component of a DNA translocating machinery. *J. Mol. Biol.* **347,** 895-902.

Alam, T. I. & Rao, V. B. (2008). The ATPase domain of the large terminase protein, gp17, from bacteriophage T4 binds DNA: implications of DNA packaging mechanism. *J. Mol. Biol.* **376**, 1272-1281.

Bamford, J. K. H. & Bamford, D. H. (1991). Large-Scale Purification of Membrane-Containing Bacteriophage PRD1 and Its Subviral Particles. *Virology* **181**, 348-352.

Bamford, D., & Mindich, L. (1982). Structure of the lipid-containing bacteriophage PRD1: disruption of wild-type and nonsense mutant phage particles with guanidine hydrochloride. *J. Virol.* **44,** 1031-1038.

Bamford, D., McGraw, T., MacKenzie, G., & Mindich, L. (1983). Identification of a protein bound to the termini of bacteriophage PRD1 DNA. *J. Virol.* **47**(2), 311-316.

Bamford, D. H., & Ackermann, H.-W. (2000). Family *Tectiviridae*, p. 111-116. *In* M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner eds., Virus Taxonomy. *Classification and Nomenclature of Viruses*. Academic Press, San Diego.

Bamford, D. H. (2005). Family *Tectiviridae*. In "*Virus Taxonomy*: VIIIth Report of International Committee on Taxonomy *of Viruses"*. (C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball, eds.) Elsevier Academic Press, San Diego: pp.81-85.

Bamford, J. K., Cockburn, J. J., Diprose, J., Grimes, J. M., Sutton, G., Stuart, D. I., Bamford, D. H. (2002). Diffraction quality crystals of PRD1, a 66-MDa dsDNA virus with an internal membrane. *J. Struct. Biol.* **139**(2), 103-112.

Bamford, J. K. H. & Butcher S. J. (2008). Icosahedral dsDNA Bacterial Viruses with an Internal Membrane. Encyclopedia of Virology (Mahy, B. W. J. & van Regenmortel, M. H. V. eds., IIIed) Academic Press.

Barenboim, M., Chang, C.-Y., dib Hajj, F. & & Young, R. (1999). Characterization of the dual start motif of class II holin gene. *Molec. Microbiol.* **32**,715-727.

Baumann, R. G. & Black, L.W. (2003). Isolation and characterisation of T4 bacteriophage gp17 terminase, a large subunit multimer with enhanced ATPase activity. *J. Biol. Chem.* **278**, 20175-81.

Bernhardt, T.G., Struck, D.K. & Young, R. (2001a). The lysis protein E of ϕX174 is a specific inhibitor of the Mra catalysed step in peptidoglycan synthesis. *J. Biol. Chem.* **276**, 6093-6097.

Bernhardt, T.G., Wang, I.-N., Struck, D.K. & Young, R. (2001b). A protein antibiotic in the phage Qβ virion: Diversity in lysis targets. *Science* **292**, 2326-2329.

Bernhardt, T.G., Wang, I.-N., Struck, D.K. & Young, R. (2002). Breaking free: "protein antibiotics" and phage lysis. *Res. Microbiol.* **153**(8), 493-501.

Black, L. W. (1981). *In vitro* packaging of bacteriophage T4 DNA. *Virology*, **113**, 336-344.

Black, L.W. & Silverman, D. (1978). Model for DNA packaging into bacteriophage T4 heads. *J. Virol.* **28**, 643-655.

Bläsi, U. & Young, R. (1990). Two beginnings for a single purpose: the dual-start holins in the regulation of phage lysis. *Molec. Microbiol.* **21**, 675-682.

Bläsi, U., Chang, C.Y., Zagotta, M.T., Nam, K.B. & Young, R. (1990). The lethal λ *S* gene encodes its own inhibitor. *EMBO J.* **9**, 981-989.

Bochkareva, E. S., Solovieva, M. S. & Girshovich, A. S. (1998). Targeting of GroEL to SecA on the cytoplasmic membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **95,** 478-483.

Butcher, S. J., Bamford, D. H. & Fuller, S. D. (1995). DNA packaging orders the membrane of bacteriophage PRD1. *EMBO J.* **14**, 6078-6086.

Caldentey J, Blanco L, Savilahti H, Bamford DH, Salas M. (1992). In vitro replication of bacteriophage PRD1 DNA. Metal activation of protein-primed initiation and DNA elongation. *Nucl. Acids Res.* **20**(15), 3971-3976.

Caldentey, J., Blanco, L., Bamford, D. H. & Salas, M. (1993). *In vitro* replication of bacteriophage PRD1 DNA. Characterization of the protein-primed initiation site. *Nucl. Acids Res.* **21**, 3725–3730.

Caldentey, J., Hänninen, A.-L. & Bamford, D. H. (1994). Gene *XV* of bacteriophage PRD1 encodes a lytic enzyme with muramidase activity. *J. Eur. Biochem.* **225,** 341-346.

Casjens, S. & Hendrix, R. (1988). Control mechanisms in dsDNA bacteriophage assembly. In: the Bacteriophages, pp. 15-91, Calendar R. (ed.), Plenum Press, NewYork.

Casjens, S. (2008). Capsid Assembly: Bacterial Virus Sructure and Assembly. Encyclopedia of Virology (Mahy, B. W. J.& van Regenmortel M. H. V. eds., IIIed) Academic Press.

Caspar, D. L. & Klug, A. (1962). Physical principles in the construction of regular viruses. *Cold Spring Harb. Symp. Quant. Biol.* **27**, 1-24.

Catalano, C. E. (2005). Viral Genome packaging machines: An overview. *In* C. E. Catalano (ed.), Viral Genome Packaging Machines: Genetics, Structure and Mechanism. Landes Bioscience/Eurekah.com and Kluwer Academic/Plenum Publishers.

Ceres, P., Stray, S. J. & Zlotnik, A. (2004). Hepatitis B virus capsid assembly is enhanced by naturally occurring mutation F97L. *J. Virol.* **78**, 9538-9543.

Cerritelli, M.E. & Studier, F.W. (1996). Assembly of T7 capsids from independently expressed and purified head protein and scaffolding protein. *J. Mol. Biol.* **258**, 286-298.

Chang, J., Weigele, P., King, J., Chiu, W. & Jiang. W. (2006). Cryo-EM asymmetric reconstruction of bacteriophage P22 reveals organization of its DNA packaging and infecting machinery. *Structure* **14**, 1073-1082.

Conway, J.F., Wikoff, W.R., Cheng, N. Duda, R.L. Hendrix R. W. Johnson, J.E. & Steven, A.C. (2001). Virus maturation involving large subunit rotations and local refolding. *Science* **292**, 744-748.

Davis, T.N., Muller, E.D. & Cronan J. E., Jr. (1982). The virion of the lipid containing bacteriophage PR4. *Virology* **120**, 287-306.

Deaton, J., Savva, C. G., Sun, J., Holzenburg, A., Berry, J. & Young, R. (2004). Solubilization and delivery by GroEL of megadalton complexes of the λ holin. Protein Science **13,**1778-1786.

Dokland, T., Bernal, R. A., Burch A., Pletnev S., Fane B. A. & Rossmann, M.G. (1999). The role of scaffolding proteins in the assembly of the small, singe-stranded DNA virus ϕX174. *J. Mol. Biol.* **288**, 595-608.

Dokland, T. (1999). Scaffolding proteins and their role in viral assembly. *Cell. Mol. Life Sci.* **56**, 580-603.

Dokland, T. (2000). Freedom and restraint: themes in virus capsid assembly. *Structure* **8**, R157-R162.

Draper, B. & Rao, V.B. (2007). An ATP hydrolysis sensor in the DNA packaging motor from bacteriophage T4 suggests an inchworm-type translocation mechanism. *J. Mol. Biol.* **369**, 79-94.

Dröge, A., Santos, M. A., Stiege, A. C., Alonso, J. C., Lurz, R., Trautner, T. A. & Tavares P. (2000). Shape and DNA packaging activity of bacteriophage SPP1 procapsid: protein components and interactions during assembly. *J. Mol. Biol.* **296,** 117-132.

Dröge, A. & Tavares, P. (2000). *In vitro* packaging of DNA of the *Bacillus subtilis* bacteriophage SPP1. *J. Mol. Biol.* **296**, 103-115.

Duda, R. L., Hempel, J., Michel, H., Shabanowitz, J., Hunt, D. & Hendrix, R.W. (1995a). Structural transition during bacteriophage HK97 head assembly. *J. Mol. Biol.* **247**, 618-635.

Duda, R. L., Martincic, K. & Hendrix, R.W. (1995b). Genetic basis of bacteriophage HK97 prohead assembly. *J. Mol. Biol.* **247**, 636-647.

Duda, R. L. (2008). Icosahedral Tailed dsDNA Bacterial Viruses. Encyclopedia of Virology (Mahy, B. W. J.& van Regenmortel M. H. V. eds., IIIed) Academic Press.

Earnshaw, W. & Casjens, S. (1980). DNA packaging by the double-stranded DNA bacteriophages. *Cell* **21**, 319-331.

Fujisawa, H. & Morita, M. (1997). Phage DNA packaging. *Genes Cells*, **2**, 537-545.

Fuller, D. N., Rickgauer, J. P., Jardine, P. J., Grimes, S., Anderson, D. L. & Smith, D. E. (2007a). Ionic effects on viral DNA packaging and portal motor function in bacteriophage ϕ 29. *Proc. Natl. Acad. Sci. USA*, **104**(27), 11245-11250.

Fuller, D. N., Raymer, D. M., Kottadiel, V. I., Rao, V. B. & Smith, D. E. (2007b). Single phage T4 DNA packaging motors exhibit large force generation, high velocity, and dynamic variability. *Proc. Natl. Acad. Sci. USA*, **104**(43), 16868-16873.

Fuller, D. N., Raymer D. M., Rickgauer, J. P., Robertson R. M., Catalano C. E., et al. (2007c). Measurements of single DNA molecule packaging dynamics in bacteriophage lambda reveal high forces, high motor processivity, and capsid transformations. *J. Mol. Biol.* **373,** 1113-1122.

Gaidelyte, A., Jaatinen, S. T., Daugelavicius, R., Bamford, J. K. & Bamford, D. H. (2005). The linear double-stranded DNA of phage Bam35 enters lysogenic host cells, but the late phage functions are suppressed. *J. Bacteriol.* **187**(10), 3521-3527.

Garcea, R.L. & Liddington, R.C. (1997). Structural biology of Polymaviruses. In: Chiu, W., Burnett, R.M., Garcea, R.L. eds. Structural biology of viruses. Oxford University Press, New York, pp. 187-208.

Garret, J., Fusselman, R., Hise, J., Chiou, L., Smith-Grillo, D., Schultz, R. & Young, R. (1981). Cell lysis by induction of cloned lambda lysis genes. *Mol. Gen. Genet.* **182**, 326-331.

Gaussier, H., Yang, Q. & Catalano, C. E. (2006). Building a virus from scratch: assembly of an infectious virus using purified components in a rigorously defined biochemical assay system. *J. Mol. Biol.* **357**, 1154-1166.

Gowen, B., Bamford, J. K. H., Bamford, D. H. & Fuller, S. D. (2003). The tailless icosahedral membrane virus PRD1 localizes the proteins involved in genome packaging and injection at a unique vertex. *J. Virol.* **77,** 7863-7871.

Grahn, A. M., Haase J., Lanka, E. & Bamford, D. H. (1997). Assembly of a functional phage PRD1 receptor depends on 11 genes of the IncP plasmid mating pair formation complex. *J. Bacteriol.* **179**(15), 4733-4740.

Grahn, A. M., J. Caldentey, J. K. H. Bamford, and D. H. Bamford. (1999). Stable packaging of phage PRD1 DNA requires adsorption protein P2, which binds to the IncP plasmid-encoded conjugative transfer complex. *J. Bacteriol.* **181,** 6689-6696.

Grahn, A. M., Haase, J., Bamford, D. H. & Lanka, E. (2000). Components of the RP4 conjugative transfer apparatus form an envelope structure bridging inner and outer membranes of donor cells: implications for related macromolecule transport systems. *J. Bacteriol.* **182**(6), 1564-1574.

Grahn, A. M., Daugelavicius, R. & Bamford, D. H. (2002). Sequential model of phage PRD1 DNA delivery: active involvement of the viral membrane. *Mol. Microbiol.* **46**, 1199-1209.

Grahn, A. M., Butcher, S.J., Bamford, J. K. H. & D. H. Bamford. (2006). PRD1 - dissecting the genome, structure and entry. The Bacteriophages. R. Calendar ed., IIed.

Graschopf, A. & Bläsi, U. (1999). Functional assembly of the lambda S holin requires periplasmic localisation of its N-terminus. *Archives of Microbiology.* **172**, 31-39.

Grimes, S. & Anderson, D. (1989). *In vitro* packaging of bacteriophage phi29 DNA restriction fragments and the role of the terminal protein gp3. *J. Mol. Biol.* **209**(1), 91-100.

Gründling, A., Bläsi, U. & R. Young. (2000a). Biochemical and genetic evidence for three transmembrane domains in the class I holin, lambda S. *J. Biol. Chem.* **275,** 769-776.

Gründling, A., Bläsi, U. & Young, R. (2000b). Genetic and biochemical analysis of dimer and oligomer interactions of the λ S holin. *J. Bacteriol.* **182**, 6082-6090.

Gründling, A., Smith, D. L., Bläsi, U.& Young, R. (2000c). Dimerization between the holin and holin inhibitor of phage lambda. *J. Bacteriol.* **182,** 6075-6081.

Gründling, A., Manson, M. D. & Young, R. (2001). Holins kill without warning. *Proc. Natl. Acad. Sci. USA* **98,** 9348-9352.

Guasch, A., Pous, J., Ibarra, B., Gomis-Ruth, F. X., Valpuesta, J. M., Sousa, N., Carrascosa, J. L. & Coll, M. (2002). Detailed architecture of a DNA translocating machine: the high-resolution structure of the bacteriophage phi29 connector particle. *J. Mol. Biol.* **315,** 663-676.

Guo, P., Grimes, S. & Anderson, D. (1986). A defined system for *in vitro* packaging of DNA-gp3 of the Bacillus subtilis bacteriophage phi 29. *Proc. Natl. Acad. Sci. USA*, **83**, 3505-3509.

Guo, P., Peterson, C. & Anderson, D. (1987). Prohead and DNA-gp3-dependent ATPase activity of the DNA packaging protein gp16 of bacteriophage phi29. *J. Mol. Biol.* **197,** 229-236.

Guo, P.X., Erikson, S., Xu, W., Olson, N., Baker, T.S. & Anderson, D. (1991). Regulation of the phage phi29 prohead shape and size by the portal vertex. *Virology* **183**, 366-373.

Guo, P. & Lee, T. J. (2007). MicroReview. Viral nanomotors for packaging of dsDNA and dsRNA. *Mol. Microbiol.* **64**(4), 886-903.

Guttman, B., Raya, R. & Kutter E. (2005). Basic Phage Biology. In Bacteriophages biology and applications. Kutter, E. & Sulakvelidze, A. eds., CRC Press.

Hendrix, R. W. (1978). Symmetry mismatch and DNA packaging in large bacteriophages. *Proc. Natl. Acad. Sci. USA*, **75**, 4779-4783.

Huang, L. P. & Guo, P. (2003). Use of PEG to acquire highly soluble DNA-packaging enzyme gp16 of bacterial virus phi29 for stoichiometry quantification. *J. Virol. Met.* **109**, 234-244.

Hugel, T., Michaelis, J., Hetherington, C. L., Jardine, P. J, Grimes, S., Walter, J. M., Falk, W., Anderson, D. L. & Bustamante, C. (2007). Experimental test of connector rotation during DNA packaging into bacteriophage phi29 capsids. *PLoS Biol*. **3**, e59.

Hänninen, A.-L., Bamford, D. H. & Bamford, J. K. H. (1997). Assembly of membrane-containing bacteriophage PRD1 is dependent on GroEL and GroES. *Virology* **227,** 207-210.

Hänninen, A.-L., Bamford, D. H. & Bamford, J. K. H. (1997). Probing the phage PRD1 specific proteins during infection by monoclonal and polyclonal antibodies. *Virology* **227,** 198-206.

Jaatinen, S. T., Viitanen, S. J., Bamford, D. H. & Bamford J. K. H. (2004). Integral membrane protein P16 of bacteriophage PRD1 stabilizes the adsorption vertex structure. *J. Virol.* **78,** 9790-9797.

Jardine, P. J., & Anderson, D. L. (2006). DNA packaging in double-stranded DNA phages, p. 49-65. *In* R. Calendar (ed.), The Bacteriophages, 2 ed. Oxford University Press (USA).

Jiang, W., Li, Z., Zhang, Z., Baker, M.L., Prevelige, P.E.J. & Chiu, W. (2003). Coat protein fold and maturation transition of bacteriophage P22 seen at subnanometer resolutions. *Nat. Struct. Biol.***10**(2), 131-135.

Johnson-Boaz, R., Chang, C.Y. & Young, R. (1994). A dominant mutation in the bacteriophage lambda S gene causes premature lysis and absolute defective plating phenotype. *Mol. Microbiol.* **13**, 495-504.

King, J. & Casjens, S. (1974). Catalytic head assembling protein in virus morphogenesis. *Nature* **251**, 112 - 119.

King, J., Griffin-Shea, R. & Fuller, M.T. (1980). Scaffolding proteins and the genetic control of virus shell assembly. *Quart. Rev. Biol.* **55**, 369-393.

Klug, A. (1999). The tobacco mosaic virus particle: structure and assembly. *Phil. Trans. R. Soc. Lond. B* **354**, 531-535.

Krupovic M, Cvirkaite-Krupovic V, Bamford DH. (2008). Identification and functional analysis of the Rz/Rz1-like accessory lysis genes in the membrane-containing bacteriophage PRD1. *Mol. Microbiol.* **68**(2), 492-503.

Lander, G. C., Tang, L., Casjens, S. R., Gilcrease, E. B., Prevelige, P., Poliakov, A., Potter, C. S., Carragher, B. & Johnson, J. E. (2006). The structure of an infectious P22 virion shows the signal for headful DNA packaging. *Science* **312,** 1791-1795.

Laurinavicius, S., Kakela, R., Somerharju, P. & Bamford, D. H. (2004). Phospholipid molecular species profiles of tectiviruses infecting Gram-negative and Gram-positive hosts. *Virology* **322**(2), 328-336.

Lebedev, A. A., Krause M. H., Isidro, A. L., Vagin, A. A., Orlova, E. V., Turner, J., Dodson, E. J., Tavares, P. & Antson, A. A. (2007). Structural framework for DNA translocation via the viral portal protein. *EMBO J.* **26**, 1984-1994.

Luo, C., Butcher, S. & Bamford, D. H. (1993). Isolation of a phospholipid-free protein shell of bacteriophage PRD1, an *Escherichia coli* virus with an internal membrane. *Virology* **194,** 564-569.

Luo, M. (2008). Assembly of viruses: Nonenveloped Particles. Encyclopedia of Virology (Mahy B. W. J., van Regenmortel M. H. V.IIIed) Academic Press.

Lurz, R., Orlova, E. V., Gunther, D., Dube, P., Droge, A., Weise, F., van Heel, M. & Tavares P. (2001). Structural organisation of the head-to-tail interface of a bacterial virus. *J. Mol. Biol.* **310,** 1027-1037.

Maluf, N., Yang, Q. & Catalano C.E. (2005). Self-association properties of the bacteriophage lambda terminase holoenzyme: implications for the DNA packaging motor. *J. Mol. Biol.* **347**, 523-542.

Mindich, L., Bamford, D., Goldthwaite, C., Laverty, M. & Mackenzie, G. (1982). Isolation of nonsense mutants of lipid-containing bacteriophage PRD1. *J. Virol.* **44,** 1013-1020.

Mindich, L., Bamford, D., McGraw, T. & Mackenzie, G. (1982). Assembly of bacteriophage PRD1: particle formation with wild-type and mutant viruses. *J. Virol.* **44**, 1021-1030.

Mitchell, M. S., Matsuzaki, S., Imai, S. & Rao, V. B. (2002). Sequence analysis of bacteriophage T4 DNA packaging/terminase genes 16 and 17 reveals a common ATPase center in the large subunit of viral terminases. *Nucl. Acids Res.* **30**(18), 4009-4021.

Moore, S. D. & Prevelige, P. E. Jr. (2002). DNA Packaging: A New Class of Molecular Motors. *Curr. Biol*. **12**, 96-98.

Morita, M., Tasaka, M. & Fyjisawa, H. (1993). DNA packaging ATPase of bacteriophage T3. *Virology* **193**, 748-752.

Muller, E.D. & Cronan, J. E. Jr. (1983). The lipid-containing bacteriophage PR4. Effectsof altered lipid composition on the virion. *J. Mol. Biol.* **165**, 109-124.

Navaratnarajah, C.K., Warrier, R., Kuhn, R.J. (2008). Assembly of viruses: Enveloped Particles. Encyclopedia of Virology (Mahy B. W. J., van Regenmortel M. H. V.IIIed) Academic Press.

Navarre, W. W., Ton-That, H., Faull, K. F. & Schneewind, O. (1999). Multiple enzymatic activities of the murein hydrolase from staphylococcal phage ϕ11. *J. Biol. Chem*. **274**, 15847-15856.

Oliveira, L., A. O. Henriques, and P. Tavares. (2006). Modulation of the viral ATPase activity by the portal protein correlates with DNA packaging efficiency. *J. Biol. Chem.* **281**, (31), 21914-21923.

Olsen, R. H., Siak, J. & Gray, R. H. (1974). Characteristics of PRD1, a plasmid-dependent broad host range DNA bacteriophage. *J. Virol.* **14**, 689-699.

Orlova, E. V., Dube, P., Beckmann, E., Zemlin, F., Lurz, R., Trautner, T. A., Tavares, P. & van Heel M. (1999). Structure of the 13-fold symmetric portal protein of bacteriophage SPP1. *Nat. Struct. Biol.* **6**, 842-846.

Orlova, E. V., Gowen, B., Droge, A., Stiege, A., Weise, F., Lurz, R., van Heel, M. & Tavares P. (2003). Structure of a viral DNA gatekeeper at 10 Å resolution by cryo-electron microscopy. *EMBO J.* **22,** 1255-1262.

Ortega, M. E., Gaussier, H. & Catalano C. E. (2007). The DNA maturation domain of gpA, the DNA packaging motor protein of bacteriophage lambda, contains an ATPase site associeted with endonuclease activity. *J. Mol. Biol.* **373**, 851-865.

Pakula, T. M., Caldentey, J., Gutiérrez, C., Olkkonen. V. M., Salas, M. & Bamford, D. H. (1993). Overproduction, purification, and characterization of DNA-binding protein P19 of bacteriophage PRD1. *Gene* **126,** 99-104.

Parent, K. N., Suhanovsky, M. M. & Teschke, C. M. (2007). Phage P22 procapsid equilibrate with free coat protein subunits. *J. Mol. Biol.* **365**, 513-22.

Park, T., Struck, D.K., Dankenbrig, C.A. & Young, R. (2007). The pinholin of lambdoid phage 21: control of lysis by membrane depolarization. *J. Bacteriol.* **189**, 9135-9139.

Prasad, B.V., Prevelige, P.E., Marietta, E., Chen, R.O., Thomas, D., King, J. & Chiu, W. (1993). Three-dimentional transformation of capsids associated with genome packaging in bacterial virus. *J. Mol. Biol.* **231**, 65-74.

Ramanculov, E. R. & Young, R. (2001). An ancient player unmasked: T4 rI encodes a *t*-specific antiholin. *Mol. Microbiol.* **41**, 575-583.

Rao, V.B. & Black, L.W. (1985). DNA packaging of bacteriophage T4 proheads in vitro. Evidence that prohead expantion is not coupled to DNA packaging. *J. Mol. Biol.* **185**, 565-578.

Rao, V. B. & Feiss M. (2008). The Bacteriophage DNA Packaging Motor. *Ann. Rev. Genet.* **42.** 19.1-19.35.

Ravantti, J. J., Gaidelyte, A., Bamford, D. H. & Bamford, J. K. (2003). Comparative analysis of bacterial viruses Bam35, infecting a gram-positive host, and PRD1, infecting gram-negative hosts, demonstrates a viral lineage. *Virology* **313,** 401-414.

Reader, R. W. & Siminovitch, L. (1971). Lysis defective mutants of bacteriophage lambda: Genetics and physiology of S cistron mutants. *Virology* **43**, 607-622.

Reader, R. W. & Siminovitch, L. (1971). Lysis defective mutants of bacteriophage lambda: on the role of the S function in lysis. *Virology* **43**, 607-622.

Ross, P. D., Conway, J. F., Cheng, N., Dierkes, L., Firek, B. A., Hendrix, R. W., Steven, A.C. & Duda, R. L. (2006). A free energy cascade with locks drives assembly and maturation of bacteriophage HK97 capsid. *J. Mol. Biol.* **364**, 512-525.

Russel, M. & Model, P. (2006). Filamentous Phage. In Bacteriophages. II edition, Calendar, R. & Abedon, S.T. Oxford university press, New York.

Rydman, P. S., Caldentey, J., Butcher, S. J., Fuller, S. D., Rutten, T. & Bamford, D. H. (1999). Bacteriophage PRD1 contains a labile receptor-binding structure at each vertex. *J. Mol. Biol.* **291,** 575-587.

Rydman, P. S., Bamford, J. K. & Bamford, D. (2001). A minor capsid protein P30 is essential for bacteriophage PRD1 capsid assembly. *J. Mol. Biol.* **313**, 785-795.

Rydman, P. S. & Bamford, D. H. (2000). Bacteriophage PRD1 DNA entry uses a viral membrane-associated transglycosylase activity. *Mol. Microbiol*. **37**(2), 356-363.

Rydman, P. S., & Bamford D. H. (2002). The lytic enzyme of bacteriophage PRD1 is associated with the viral membrane. *J. Bacteriol.* **184,** 104-110.

Rydman, P. S. & Bamford, D. H. (2003). Identification and mutational analysis of bacteriophage PRD1 holin protein P35. *J. Bacteriol.* **185,** 3795-803.

San Martin, C., Burnett, R. M., de Haas, F., Heinkel, R., Rutten, T., Fuller, S. D., Butcher, S. J. & Bamford D.H. (2001). Combined EM/X-ray imaging yields a quasi-atomic model of the adenovirus-related bacteriophage PRD1 and shows key capsid and membrane interactions. *Structure* **9**(10), 917-930.

Sao-Jose, C., Parreira, R., Vieira, G. & Santos, M. A. (2000). The N-terminal region of the *Oenococcus oeni* bacteriophage fOg44 lysin behaves as a bona fide signal peptide in *Escherichia coli* and as a *cis*-inhibitory element, preventing lytic activity on Oenococcal cells. *J. Bacteriol.* **182,** 5823-5831.

Savilahti, H., Caldentey, J., & Bamford, D. H. (1989). Bacteriophage PRD1 terminal protein: expression of gene VIII in Escherichia coli and purification of the functional P8 product. *Gene* **85**(1), 45-51.

Savilahti, H., Caldentey, J., Lundström, K., Syväoja, J. E. & Bamford, D. H. (1991). Overexpression, purification, and characterization of *Escherichia coli* bacteriophage PRD1 DNA polymerase. *In vitro* synthesis of full-length PRD1 DNA with purified proteins. *J. Biol. Chem.* **266,** 18737-18744.

Savva, C. G., Dewey, J. S., Deaton, J., White, R. L., Struck, D. K., Holzenburg, A. & Young, R. (2008). The holin of bacteriophage lambda forms rings with large diameter. *Mol. Microbiol.* **69**(4),784-793.

Simpson, A. A., Leiman, P. G., Tao, Y., He, Y., Badasso, M. O., Jardine, P. J., Anderson, D.L. & Rossmann M. G. (2001). Structure determination of the head-tail connector of bacteriophage Phi29. *Acta Crystallographica* Section D **57,** 1260-1269.

Simpson, A., Tao, Y., Leiman, P., Badasso, M., He, Y. et al. (2000). Structure of bacteriophage phi29 DNA packaging motor. *Nature* **408**, 745-750.

Smith, D. E., Tans, S. J., Smith, S. B., Grimes, S., Anderson, D. L. & Bustamante, C. (2001). The Bacteriophage phi29 portal motor can package DNA against a large internal force. *Nature,* **413**, 748-752.

Steiner, M., Lubitz, W. & Bläsi, U. (1993). The missing link in phage lysis of gram-positive bacteria: gene 14 of *Bacillus subtilis* phage ϕ29 encodes the functional homolog of lambda S protein. *J. Bacteriol.* **175**, 1038-1042.

Steven, A.C., Heymann, B. J., Cheng, N., Trus, B.L. & Conway, J.F. (2005). Virus maturation: dynamics and mechanism of a stabilizing structural transition that leads to infectivity. *Curr. Op. Stru. Biol.* **15**, 227-236.

Stockley, P. G. (2008). Icosahedral ssRNA Bacterial Viruses. Encyclopedia of Virology (Mahy, B. W. J. & van Regenmortel M. H. V. eds., IIIed) Academic Press.

Strömsten, N. J., Bamford, D. H. & Bamford, J. K. (2005). *In vitro* DNA packaging of PRD1: a common mechanism for internal-membrane viruses. *J. Mol. Biol.* **348,** 617-629.

Strömsten, N. J., Bamford, D. H. & Bamford, J. K. H. (2003). The unique vertex of bacterial virus PRD1 is connected to the viral internal membrane. *J. Virol.* **77,** 6314-6321.

Summer, E.J.,Berry, J., Tran, T.A.T.,Niu, L., Struck, D.K. & Young, R. (2007). *Rz/Rz1* Lysis Gene Equipments in Phages of Gram-negative Hosts. *J. Mol. Biol.* **373**, 1098-1112.

Sun, S., Kondabagil, K., Gentz, P. M., Rossmann, M. G. & Rao, V. B. (2007). The structure of the ATPase that powers DNA packaging into bacteriophage T4 Procapsids. *Mol. Cell* **25**, 943-949.

Thuman-Commike, P. A., Greene, B., Malinski, J. A., King, J. & Chiu, W. (1998). Role of the scaffolding protein in P22 procapsid size determination suggested by T=4 and T=7 procapsid structures. *Biophys. J.* **74**(1), 559-568.

Voyles B.A., (2002). The biology of viruses. II ed., McGraw-Hill higher Education.

Walderich, B. & Höltje, J.-V. (1989). Specific localization of the lysis protein of bacteriophage MS2 in membrane adhesion sites of *Escherichia coli. J. Bacteriol.* **171**, 3331-3336.

Walderich, B., Ursinus-Wöosner, A., van Duin, J. & Höltje, J.-V. (1988). Introduction of the autolytic system of *Escherichia coli* by specific insertion of bacteriophage MS2 lysis protein into the bacterial cell envelope. *J. Bacteriol.* **170,** 5027-5033.

Walker, J.T. & Walker, D. H. (1980). Mutations in coliphage P1 affecting host cell lysis. *J. Virol.* **35**, 519-530.

Walker, J.E., Saraste, M., Runswick, M. J. & Gay, N.J. (1982). Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **8**, 945-951.

Wang, I.-N., Dykhuizen, D. E. & Slobodkin, L. B. (1996). The evolution of phage lysis timing. *Evolutionary Ecology* **10**, 545-558.

Wang, I.-N., Smith, D. L. & Young, R. (2000). Holins: protein clocks of bacteriophage infections. *Ann. Rev. Microbiol.* **54,** 799-825.

Wang, I.-N., Deaton, J. & Young, R. (2003). Sizing the holin lesion with an endolysin-β-galactosidase fusion. *J. Bacteriol.* **185,** 779-787.

Wang, I.-N. (2006). Lysis timing and bacteriophage fitness. *Genetics* **172**, 17-26.

Wang, S, Chang, J.R. & Dokland, T. (2006). Assembly of bacteriophage P2 and P4 procapsids with internal scaffolding protein. *Virology* **346**, 133-140.

Wong, F. H. & Bryan, L. E. (1978). Characteristics of PR5, a lipid-containing plasmid-dependent phage. *Canad. J. Microbiol.* **24**, 875-882.

Xu, M., Struck, D. K., Deaton, J., Wang, I.-N. & Young, R. (2004). A signal-arrest-release sequence mediates export and control of the phage P1 endolysin. *PNAS* **101**, 6415-6420.

Xu, M., Arulandu, A., Struck, D.K.,Swanon, S., Sacchenttini, J. C. & Young, R. (2004). Disulfide izomerization after membrane release of its SAR domain activates P1 lysozyme. *Science* **307**, 113-117.

Yang, Q. & Catalano, C. E. (2003). Biochemical characterization of bacteriophage lambda genome packaging *in vitro*. *Virology*, **305**, 276-287.

Young, R., Way, J., Way, S., Yin, J., & Syvanen, M. (1979). Transposition mutagenesis of phage lambda: a new gene affecting cell lysis. *J. Mol. Biol.* **132**, 307-322.

Young, R. (1992). Bacteriophage lysis: mechanism and regulation. *Microbiol. Reviews.* **56,** 430-481.

Young, R. & Bläsi, U. (1995). Holins: form and function in bacteriophage lysis. *FEMS Microbiol. Rev.* **17**, 191-205.

Young, R., Wang, I.-N., Roof, W. D. (2000). Phages will out: strategies of host cell lysis. *Trends in Microbiology* **8,** 120-128.

Young, R. (2002). Bacteriophage holins: deadly diversity. *J. Mol. Microbiol. Biotechnol.* **4**(1), 21-36.

Young, R. & Wang I.-N. (2006). Phage Lysis. In Bacteriophages. II edition, Calendar, R. & Abedon, S.T. Oxford university press, New York.

Zheng, Y., Struck, D. K., Dankenbrig, C. A. & Young, R. (2008). Evolutionary dominance of holin lysis systems derives from superior genetic malleability. *Microbiol.* **154**, 1710-1718.

Ziermann, R., Bartlett, B., Calendar, R. & Christie, G. E. (1994). Funcions involved in bacteriophage P2-induced host cell lysis and identification of the new tail gene. *J. Bacteriol.* **176,** 4974-4984.

Zlotnik, A. (2007). Distinguishing reversible from irreversible virus capsid assembly. *J. Mol. Biol.* **366**, 14-18.